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(54) Title: TARGETED GENE MODIFICATION BY PARVOVIRAL VECTORS

(57) Abstract

This invention provides methods for obtaining targeted gene modification in vertebrate cells using parvoviruses, including adeno–associated virus (AAV). The parvoviral vectors used in the claimed methods are capable of targeting a specific genetic modification to a preselected target locus in a cellular genome by homologous pairing.

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TARGETED GENE MODIFICATION BY PARVOVIRAL VECTORS

BACKGROUND OF THE INVENTION

Field of the Invention

This invention pertains to the field of targeted modification of cellular DNA in vertebrate cells by homologous pairing using parvoviral vectors, including vectors based on adeno-associated virus (AAV).

Background

Although the development of integrating vectors based on eukaryotic viruses made possible the efficient introduction of genes into mammalian chromosomes, there are many situations where it would be preferable to modify specific chromosomal sequences, thereby eliminating unwanted chromosomal genotypes and avoiding position effects on gene expression. This is especially true in gene therapy, where mutant genes can have dominant effects and tissue-specific controls on expression are often critical.

Previously known methods for introducing defined mutations into mammalian chromosomes by gene targeting involve transfection, electroporation or microinjection (Smithies *et al.* (1985) *Nature* 317: 230-234; Thomas *et al.* (1986) *Cell* 44: 419-428). These methods, except for microinjection, produce homologous recombination events in only a small fraction of the total cell population, on the order of 10^{-6} in the case of mouse embryonic stem cells (Doetschman *et al.* (1987) *Nature* 330: 576-578; Thomas and Capecchi (1987) *Cell* 51: 503-512). Thus, the routine use of these methods requires preselection of transformed cells, making it difficult to apply the techniques to normal cells and *in vivo* applications. Microinjection is likewise not feasible for routine use because each cell must be injected individually using a time-consuming, labor-intensive procedure.

Attempts to use transducing viral vectors to overcome these limitations and achieve chromosomal gene targeting experiments have been performed with retroviral and adenoviral vectors, but the results were not significantly better than can be obtained by

transfection, with homologous recombination occurring in 10^{-5} to 10^{-6} cells (Ellis and Bernstein (1989) *Mol. Cell. Biol.* 9: 1621-1627; Wang and Taylor (1993) *Mol. Cell. Biol.* 13: 918-927).

Adeno-associated virus 2 (AAV) is a 4.7 kb single stranded DNA virus that has been developed as a transducing vector capable of integrating into mammalian chromosomes (Muzyczka (1992) *Curr. Top. Microbiol. Immunol.* 158: 97-129). Two thirds of integrated wild-type AAV proviruses are found at a specific human chromosome 19 site, 19q13-qter (Kotin *et al.* (1991) *Genomics* 10: 831-834; Kotin *et al.* (1990) *Proc. Nat'l. Acad. Sci. USA* 87: 2211-2215; Samulski *et al.* (1991) *EMBO J.* 10: 3941-3950). The site-specific integration event is a non-homologous recombination reaction that appears to be mediated by the viral Rep protein (Giraud *et al.* (1995) *J. Virol.* 69: 6917-6924; Linden *et al.* (1996) *Proc. Nat'l. Acad. Sci. USA* 93: 7966-7972). While this feature could prove useful in some applications, AAV vectors with deletions in the viral *rep* gene have not been found to integrate at this same locus (Russell *et al.* (1994) *Proc. Nat'l. Acad. Sci. USA* 91: 8915-8919; Walsh *et al.* (1992) *Proc. Nat'l. Acad. Sci. USA* 89: 7257-7261). Southern analysis of integrated *rep*⁻ AAV vector proviruses suggests that integration sites are random (Lebkowski *et al.* (1988) *Mol. Cell. Biol.* 8: 3988-3996; McLaughlin *et al.* (1988) *J. Virol.* 62: 1963-1973; Russell *et al.* (1994) *supra.*; Walsh *et al.* (1992) *supra.*) and sequencing of integrated vector junction fragments has confirmed that integration occurs by non-homologous recombination at a variety of chromosomal sites.

Thus, a need exists for methods of obtaining specific genetic modification at selected target sites in vertebrate cellular genomes at high frequencies. The present invention fulfills this and other needs.

SUMMARY OF THE INVENTION

The claimed invention provides methods of producing a vertebrate cell that has a modification at a pre-selected target locus. The methods involve contacting the cell with a parvoviral vector that has a recombinant viral genome which includes a targeting construct that includes a DNA sequence which is substantially identical to the target locus except for the modification being introduced. The recombinant viral genome is allowed to enter the vertebrate cell by transduction, which results in the modifications being introduced into the target locus as a result of homologous pairing between the targeting construct and

the target locus. The modification can include one or more deletions, insertions, substitutions, or a combination thereof. The methods can be used for introducing a second modification at a second target locus by transducing a cell with a parvoviral vector that has a second targeting construct that is at least substantially identical to the second target locus except for the second modification. Additional target loci can be modified by transduction using parvoviral vectors that have appropriate targeting constructs.

Also provided by the invention are vertebrate cells that contain specific genetic modifications at a preselected target locus that were introduced into the cells, or ancestors of the cells, by contacting the cells with a parvoviral vector that has a recombinant viral genome which includes a targeting construct that includes a DNA sequence which is substantially identical to the target locus except for the modification being introduced. These cells can be cultured *in vitro*, *ex vivo*, or can be part of an organism.

In another embodiment, the invention provides methods for making transgenic and chimeric animals that have site-specific genetic modifications at a predetermined target locus, as well as transgenic and chimeric animals produced using these methods.

The invention also provides methods for introducing a modification of a target locus in a cell in a vertebrate by contacting a cell *ex vivo* with a parvoviral vector having a recombinant viral genome that includes a targeting construct that includes a DNA sequence that is, except for the modification being introduced, at least substantially identical to the target locus. The recombinant viral genome is allowed to enter the cell by transduction, after which homologous pairing occurs between the targeting construct and the target locus resulting in the modifications being introduced into the cellular DNA at the target locus. The transduced cell is then introduced into a vertebrate.

In another embodiment, the invention provides methods for making a modification of a target locus in a cell in a vertebrate by administering to the vertebrate a parvoviral vector. The parvoviral vectors used in these methods have a recombinant viral genome that includes a targeting construct that includes a DNA sequence that is, except for the modification being introduced, at least substantially identical to the target locus. The recombinant viral genome is allowed to enter the cell by transduction, after which

homologous pairing occurs between the targeting construct and the target locus resulting in the modifications being introduced into the cellular DNA at the target locus.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1A is a diagram of the adeno-associated viral vector AAV-SNori, which is described in Example 1. This vector includes two AAV terminal repeats (TR), a bacterial gene encoding neomycin phosphotransferase (Neo) under the control of an SV40 early promoter and a bacterial Tn5 promoter, a p15A plasmid replication origin, and a eukaryotic polyadenylation site. Also shown are the vectors AAV-SNO39 and AAV-SNO648, which contain mutations at bp 39 and bp 648 of the *neo* gene, respectively. Figure 1B is an autoradiogram which shows the results of a Southern blot analysis of *Bam*HI-digested genomic DNA from G418-resistant HeLa cell clones that had been modified as described in Example 1. The lane captioned "HeLa" shows hybridization of a *neo* gene probe to genomic DNA from unmodified HeLa cells, the lane captioned "HSNO39" shows hybridization of the probe to genomic DNA from HSNO39 cells that contain three copies of a plasmid that contained the internal portion of AAV-SNO39, and lanes 1-11 show hybridization of the probe to eleven different clones obtained by modifying HSNO39 cells using the parvoviral vector AAV-SNO648.

Figure 2A is a diagram of the human *HPRT* locus, as well as the AAV vectors HPe2/3 and HPe2/3X, which were used to modify the human *HPRT* locus. In addition to the indicated portion of the *HPRT* gene, these vectors, which are described in Example 2, contain two AAV terminal repeats (TR), and four Alu repeats designated O, P, Q, and R. Figures 2B and 2C are autoradiograms of HT-1080 human fibrosarcoma cell genomic DNA that had been digested with *Hin*dIII (Figure 2B) or *Hin*dIII plus *Pvu*I (Figure 2C). The lanes captioned "HT1080" shows hybridization of the probe shown in Figure 2A to genomic DNA from unmodified HT1080 cells, and lanes 1-13 show hybridization of this probe to genomic DNA from thirteen different clones that were made 6TG resistant by transduction using the AAV vector AAV-HPe2/3X.

Figure 3 shows the results of an experiment, described in Example 2, in which AAV vectors AAV-HPe2/3 and AAV-HPe2/3X were used to modify the *HPRT* locus in HT-1080 cells. The percent of 6TG-resistant cells obtained is shown.

Figure 4 presents the results of an analysis of the effect of multiplicity of infection on the frequency with which a defective *neo* gene present in HeLa cells is corrected by the AAV vector AAV-SNO648. This experiment is described in Example 3.

Figure 5 shows a comparison of the frequency of *neo* gene correction in HSNO39 cells obtained using transduction versus transfection as described in Example 4. Transduction was carried out using the AAV vector AAV-SNO648, while transfection was performed using the plasmid pASNO648 (which contains the entire AAV-SNO648 genome), pASNO39 (which contains a mutation at base pair 39 of the *neo* gene) and pASNori2 (which has no *neo* mutation).

Figure 6 shows the fraction of normal human fibroblasts having a modified HPRT gene after transduction using the AAV vector AAV-HPe2/3X as described in Example 5. The fraction of HPRT-modified cells is plotted versus the number of infecting AAV genomes per cell.

Figure 7 presents the results of experiments in which four different normal human fibroblast cultures were transduced with either AAV-HPe2/3 (wild-type HPRT gene) or AAV-HPe2/3X (which introduces a frameshift mutation in the HPRT gene). The percent of HPRT gene modification is shown.

DETAILED DESCRIPTION

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described. For purposes of the present invention, the following terms are defined below.

The term "cell line," as used herein, refers to individual cells, harvested cells, and cultures containing the cells, so long as they are derived from cells of the cell line referred to. A cell line is said to be "continuous," "immortal," or "stable" if the line remains viable over a prolonged time, typically at least about six months. To be considered a cell line, as used herein, the cells must remain viable for at least 50 passages. A "primary

cell," or "normal cell," in contrast, refers to cells that do not remain viable over a prolonged time in culture.

The term "cis-active nucleic acid" refers to a nucleic acid subsequence that encodes or directs the biological activity of a nucleic acid sequence. For instance, cis-active nucleic acid includes nucleic acid subsequences necessary for modification of a nucleic acid sequence in a host chromosome, nucleic acid subsequences which encode transcription factors or which direct replication or packaging of the full-length nucleic acid sequence, nucleic acid subsequences which encode structural proteins necessary for encapsidation of the nucleic acid sequence, and origins of nucleic acid replication.

The term "constitutive promoter" refers to a promoter that is active under most environmental and developmental conditions.

The term "equivalent conditions" refers to the developmental, environmental, growth phase, and other conditions that can affect a cell and the expression of particular genes by the cell. For example, where inducibility of gene expression by a hormone is being examined, two cells are under equivalent conditions when the level of hormone is approximately the same for each cell. Similarly, where the cell cycle specificity of expression of a gene is under investigation, two cells are under equivalent conditions when the cells are at approximately the same stage of the cell cycle.

The term "exogenous" as used herein refers to a moiety that is added to a cell, either directly or by expression from a gene that is not present in wild-type cells. Included within this definition of "exogenous" are moieties that were added to a parent or earlier ancestor of a cell, and are present in the cell of interest as a result of being passed on from the parent cell. "Wild-type," in contrast, refers to cells that do not contain an exogenous moiety. "Exogenous DNA," as used herein, includes DNA that has one or more deletions, point mutations, and/or insertions, or combinations thereof, compared to DNA in the wild-type target cell.

The term "homologous pairing," as used herein, refers to the pairing that can occur between two nucleic acid sequences or subsequences that are complementary, or substantially complementary, to each other. Two sequences are substantially complementary to each other when one of the sequences is substantially identical to a nucleic acid that is complementary to the second sequence, as defined below.

The term "host cell" or "target cell" refers to a cell to be transduced with a specified vector. The cell is optionally selected from *in vitro* cells such as those derived from cell culture, *ex vivo* cells, such as those derived from an organism, and *in vivo* cells, such as those in an organism.

The term "identical" in the context of two nucleic acid or polypeptide sequences refers to the residues in the two sequences which are the same when aligned for maximum correspondence. Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith and Waterman (1981) *Adv. Appl. Math.* 2: 482, by the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443, by the search for similarity method of Pearson and Lipman (1988) *Proc. Natl. Acad. Sci. USA* 85: 2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by inspection.

An indication that two nucleic acid sequences are "substantially identical" is that the polypeptide which the first nucleic acid encodes is immunologically cross reactive with the polypeptide encoded by the second nucleic acid. Another indication that two nucleic acid sequences are substantially identical is that the two molecules and/or their complementary strands hybridize to each other under stringent conditions.

The phrase "hybridizing specifically to," refers to the binding, duplexing, or hybridizing of a molecule only to a particular nucleotide sequence under stringent conditions when that sequence is present in a complex mixture (*e.g.*, total cellular) DNA or RNA. The term "stringent conditions" refers to conditions under which a probe will hybridize to its target subsequence, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (Tm) for the specific sequence at a defined ionic strength and pH. The Tm is the temperature (under defined ionic strength, pH, and nucleic acid concentration) at which 50% of the probes complementary to the target sequence hybridize to the target sequence at equilibrium. (As the target sequences are generally present in excess, at Tm, 50% of the probes are occupied at equilibrium). Typically, stringent conditions will be those in which the salt concentration is less than about 1.0 M sodium ion, typically about 0.01 to

1.0 M sodium ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (*e.g.*, 10 to 50 nucleotides) and at least about 60°C for long probes (*e.g.*, greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Specific hybridization can also occur within a living cell.

An "inducible" promoter is a promoter which is under environmental or developmental regulation.

The term "labeled nucleic acid probe" refers to a nucleic acid probe that is bound, either covalently, through a linker, or through ionic, van der Waals or hydrogen "bonds" to a label such that the presence of the probe may be detected by detecting the presence of the label bound to the probe.

The term "label" refers to a moiety that is detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful labels include ^{32}P, ^{35}S, fluorescent dyes, electron-dense reagents, enzymes (*e.g.*, as commonly used in an ELISA), biotin, dioxigenin, or haptens and proteins for which antisera or monoclonal antibodies are available.

The term "nucleic acid" refers to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, encompasses known analogues of natural nucleotides that hybridize to nucleic acids in manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence includes the complementary sequence thereof.

The term "operably linked" refers to functional linkage between a nucleic acid expression control sequence (such as a promoter, or array of transcription factor binding sites) and a second nucleic acid sequence, wherein the expression control sequence directs transcription of the nucleic acid corresponding to the second sequence.

The term "recombinant parvoviral vector" refers to a vector derived from a parvovirus that carries non-viral DNA in addition to viral DNA. The recombinant viral genome will typically include at least one targeting construct.

The term "replicating cell" refers to a cell that is passing through the cell cycle, including the S and M phases of DNA synthesis and mitosis.

The term "subsequence" in the context of a particular nucleic acid sequence refers to a region of the nucleic acid equal to or smaller than the specified nucleic acid.

A "target locus," as used herein, refers to a region of a cellular genome at which a genetic modification is desired. The target locus typically includes the specific nucleotides to be modified, as well as additional nucleotides on one or both sides of the modification sites.

A "targeting construct" refers to a DNA sequence that is present in the genomes of the recombinant parvoviral vectors used in the claimed methods and includes a region that is identical to, or substantially identical to, a region of the target locus, except for the modification or modifications that are to be introduced into the host cell genome at the target locus. The modification can be at either end of the targeting construct, or can be internal to the targeting construct. The modification can be one or more deletions, point mutations, and/or insertions, or combinations thereof, compared to DNA in the wild-type target cell.

The term "transduction" refers to the transfer of genetic material by a recombinant viral vector to a recipient cell.

A cell that has received viral vector DNA, thereby undergoing genetic modification is referred to herein as a "transduced cell," as are progeny and other descendants of such cells.

The term "transgenic" refers to a cell that includes a specific modification that was introduced into the cell, or an ancestor of the cell. Such modifications can include one or more point mutations, deletions, insertions, or combinations thereof. When referring to an animal, the term "transgenic" means that the animal includes cells that are transgenic. An animal that is composed of both transgenic and non-transgenic cells is referred to herein as a "chimeric" animal.

The term "vector" refers to a composition for transferring a nucleic acid (or nucleic acids) to a host cell. A vector comprises a nucleic acid encoding the nucleic acid to be transferred, and optionally comprises a viral capsid or other materials for facilitating entry of the nucleic acid into the host cell and/or replication of the vector in the host cell (*e.g.*, reverse transcriptase or other enzymes which are packaged within the capsid, or as part of the capsid).

The term "viral vector" refers to a vector that comprises a nucleic acid and viral capsid and/or replication functions.

Description of the Preferred Embodiments

The claimed invention provides methods of producing a vertebrate cell that has a specific modification of a target locus, and genetically modified cells produced using these methods. The methods involve the use of a recombinant parvoviral vector that is capable of targeting a genetic modification to a particular target locus at a high frequency by homologous pairing. The recombinant viral genomes of the parvoviral vectors used in the methods contain a targeting construct that includes a DNA sequence that incorporates the desired modifications as well as a DNA sequence that is substantially identical to a region of the target locus at which a modification is desired. The cell is contacted with the parvoviral vector, which transduces the recombinant viral genome into the cell, resulting in homologous pairing between the targeting construct and the target locus and concomitant introduction of the specific genetic modifications into the target locus.

The claimed methods make possible precise modifications of the genome of a cell. This allows one to avoid undesired effects that can occur when other methods of modifying a genome are used, such as disruption of a desirable gene by insertion of an exogenous gene. Moreover, one can achieve precise changes in a gene or a control region, making possible the correction of an endogenous gene without having to insert a correct copy of the gene elsewhere in the genome. The methods avoid the frequently observed "position effect" in which the level of expression of an exogenous gene is highly dependent upon the location in a cell's genomic DNA at which the exogenous gene becomes integrated. The methods also make possible the modification of genes that are too large to be introduced into cells by other methods. Rather than having to introduce an entire copy of the gene that includes the desired modifications, one can use the claimed methods to modify only a desired portion of the gene.

The claimed methods use recombinant parvoviral vectors to insert DNA that includes desired genetic modifications into the vertebrate cells to be modified. A general introduction to human parvoviruses is found, *e.g.*, in Pattison (1994) *Principles and Practice of Clinical Virology* (Chapter 23) Zuckerman *et al.* eds, John Wiley & Sons Ltd., and also in Berns (1991) "Parvoviridae and their Replication," In *Fundamental Virology*, Fields, Ed.,

Raven Press, New York, pp. 817-837, as well as references cited in each. The best characterized of the human parvoviruses are B19 and AAV, both of which are used as the basis for cell transduction vectors, *e.g.*, for gene therapy. Other parvoviral vectors that can be used include, but are not limited to, the viruses LuIII (Maxwell *et al.* (1993) *Human Gene Ther.* 4: 441-450) and minute virus of mice (mvm) (Russell *et al.* (1992) *J. Virol.* 66: 2821-2828.

In a preferred embodiment, the methods use an adeno-associated virus (AAV). AAVs are single-stranded, replication-defective DNA viruses with a 4.7 kb genome. Adeno-associated viruses are readily obtained, and their use as vectors for gene delivery was described in, for example, Muzyczka (1992) *Curr. Top. Microbiol. Immunol.* 158: 97-129, US Patent No. 4,797,368, and PCT Application WO 91/18088. Samulski (1993) *Current Opinion in Genetic and Development* 3: 74-80 and the references cited therein provides an overview of the AAV life cycle. For a general review of AAVs and of the adenovirus or herpes helper functions *see*, Berns and Bohensky (1987) *Advances in Virus Research*, Academic Press., 32: 243-306. The genome of AAV is described in Srivastava *et al.* (1983) *J. Virol.*, 45: 555-564. Carter *et al.*, U.S. Patent No. 4,797,368, describe many of the relevant design considerations for constructing recombinant AAV vectors. *See also*, Carter WO 93/24641. Additional references describing AAV vectors include, for example, West *et al.* (1987) *Virology* 160: 38-47; Kotin (1994) *Human Gene Therapy* 5:793-801; and Muzyczka (1994) *J. Clin. Invest.* 94: 1351. Construction of recombinant AAV vectors is also described in a number of additional publications, including Lebkowski, U.S. Pat. No. 5,173,414; Lebkowski *et al.* (1988) *Mol. Cell. Biol.* 8: 3988-3996; Tratschin *et al.* (1985) *Mol. Cell. Biol.* 5(11):3251-3260; Tratschin *et al.* (1984) *Mol. Cell. Biol.*, 4: 2072-2081; Hermonat and Muzyczka (1984) *Proc. Nat'l. Acad. Sci. USA*, 81: 6466-6470; McLaughlin *et al.* (1988) and Samulski *et al.* (1989) *J. Virol.*, 63: 03822-3828. AAV is a defective human parvovirus, meaning that the virus is capable of replicating and forming virus particles only in cells that are also infected with a helper virus. To obtain integration of an AAV genome into a mammalian cell, the cell is infected with the AAV in the absence of a helper virus.

Parvoviral genomes have an inverted terminal repeat sequence (ITR) at each end. For use in the claimed methods, the recombinant parvoviral vector genomes will typically have all or a portion of at least one of the ITRs or a functional equivalent, which is

generally required for the parvoviral vectors to replicate and be packaged into parvovirus particles. Both ITRs are often present in the recombinant parvoviral vector DNAs used in the claimed methods.

The recombinant viral genomes of the parvoviral vectors used in the claimed methods for genetically modifying vertebrate cells will also include a targeting construct that, except for the desired modification, is identical to, or substantially identical to, the target locus at which genetic modification is desired. The targeting construct will generally include at least about 20 nucleotides, preferably at least about 100, and more preferably about 1000-5000 nucleotides or more, that are identical to, or substantially identical to, the nucleotide sequence of a corresponding region of the target locus. By "substantially identical" is meant that this portion of the targeting construct is at least about 80% identical; more preferably, at least about 90%, and most preferably at least about 99% identical to the corresponding region of the target locus.

The targeting construct will also include the genetic modifications that are to be introduced into the target locus. The modifications can include one or more insertions, deletions, or point mutations, or combinations thereof, relative to the DNA sequence of the target locus. For example, to modify a target locus by introducing a point mutation, the targeting construct will include a DNA sequence that is at least substantially identical to the target locus except for the specific point mutation to be introduced. Upon transduction of the recombinant viral genome into the cell, homologous pairing occurs between the portions of the targeting construct that are substantially identical to the corresponding regions of the target locus, after which the DNA sequence of the targeting construct replaces that of the target locus.

A targeting construct can have the genetic modifications at either end, or within the region of the targeting construct that is identical to, or substantially identical to, the target locus. To delete a portion of a target locus, for example, the genetic modification will generally be within the targeting construct, being flanked by two regions of substantial identity to the target locus. Homologous pairing between the two regions of substantial identity and their corresponding regions of the target locus result in the sequence of the targeting construct, including the deletion, becoming incorporated into the target locus. Deletions can be precisely targeted to a desired location by this method. Similarly, genetic

modifications that involve site-specific insertion of DNA sequences into the target locus can be made by use of a targeting construct that has the DNA sequence to be inserted flanked by or next to regions of substantial identity to the target locus. Homologous pairing between the targeting construct and the corresponding regions of the target locus is followed by incorporation of the insertion sequence into the target locus.

The claimed methods can be used to introduce modifications at more than one target locus. For example, to introduce one or more modifications at a second target locus in a cellular genome, the cell can be contacted with a parvoviral vector that has a recombinant viral genome that has a targeting construct that is at least substantially identical to the second target locus, except for the desired modification or modifications. The targeting construct for the second target locus can be present in the same parvoviral vector as the targeting construct for the first target locus, or can be present in a second parvoviral vector. Where the first and second targeting constructs are present in different parvoviral vectors, the cells can be transduced with the vectors either sequentially or simultaneously. To obtain modifications at more than two target loci, this process is simply repeated as desired.

Structural genes, regulatory regions, and other sequences within the genomic DNA of a vertebrate cell are amenable to modification using the claimed methods. For example, one can introduce specific changes within structural genes that can alter the gene product of the gene, or prevent the gene product from being expressed. In this embodiment, the recombinant viral genome can include a targeting construct that is identical to, or substantially identical to, the target locus, with the exception of the specific nucleotide changes to be introduced. Homologous pairing between the targeting construct and the target locus in the cellular DNA results in the modifications present in the targeting construct becoming incorporated into the target locus. Where the gene product is a polypeptide, for example, one can use the claimed methods to obtain a gene that encodes a polypeptide having one or more specific amino acid substitutions, insertions, or deletions compared to the polypeptide encoded by the native gene. The claimed methods allow one to replace a codon that encodes an amino acid that results in the polypeptide being inactive, or less active than desired, with a codon specifies an amino acid that restores normal activity to the polypeptide. Many genetic diseases that are characterized by one or more mutations that result in amino acid changes are correctable using the claimed methods. As another

example, a target region can be modified by substituting a codon that specifies a glycosylation site for a codon that encodes an amino acid that is not part of a glycosylation site, or vice versa. A protease cleavage site can be created or destroyed, as yet another example. A nonsense codon present in the target locus can be changed to a sense codon, or where disruption of the polypeptide is desired, one can introduce a nonsense mutation into the target locus. One can obtain a fusion protein by incorporating into the targeting construct an exogenous DNA that codes for the portion of the fusion protein that is to be joined to an endogenous protein; the exogenous DNA will be in the proper reading frame for translation of the fusion protein upon incorporation of the DNA sequence of the targeting construct into the cellular genome at the target locus.

Similarly, where the gene product is a nucleic acid, the methods can be used for modification of the gene products. RNA genes that can be modified using the claimed methods, including tRNAs, ribosomal RNAs, ribozymes, telomerase subunits, and the like. Alternatively, the methods can be used to construct a gene for which the gene product consists of an endogenous nucleic acid linked to an exogenous nucleic acid. For example, an exogenous DNA that encodes a catalytic RNA can be linked to an endogenous gene. The RNA that is transcribed from this fusion gene could hybridize to endogenous nucleic acids that are substantially complementary to the endogenous portion of the fusion gene, after which the portion of the hybrid ribozyme that is expressed from the exogenous DNA can catalyze its usual reaction. Thus, the fusion gene obtained using the claimed methods provides a means for targeting a ribozyme.

The claimed methods also are useful for substituting, deleting or inserting nucleotides that make up regulatory regions that are involved in expressing a gene of interest. The altered regulatory region can change the expression of the gene by, for example, increasing or decreasing the level of expression of the gene compared to the level of expression under equivalent conditions in an unmodified cell. The modifications can, for example, result in expression of the gene under situations where the gene would not typically be expressed, or can prevent expression of a gene that normally would be expressed under particular circumstances. One can use the claimed methods to insert a heterologous transcription control element, or modify an endogenous control element, such as a promoter, enhancer, transcription termination signal, at a location relative to the gene of interest that is

appropriate for influencing expression of the gene. By replacing a constitutive promoter with an inducible promoter, for instance, one can tie expression of the gene to the presence or absence of a particular environmental or developmental stimulus. Similarly, regions that are involved in post-transcriptional modification, such as RNA splicing, polyadenylation, translation, as well as regions that code for amino acid sequences involved in post-translational modification can be inserted, deleted, or modified. Examples of transcription control elements that can be modified or replaced using the claimed methods include, but are not limited to, response elements, promoters, enhancers, locus control regions, other transcription initiation signals, transcription elongation signals, introns, RNA stability sequences, transcription termination signals, polyadenylation sites, and splice sites. Expression of a gene can also be modulated by using the claimed methods to introduce or destroy DNA methylation sites.

In one embodiment, the claimed methods are used to obtain selective expression of a nucleic acid in a cell. Selective expression of a nucleic acid refers to the ability of the nucleic acid to be expressed in a desired cell type and/or under desired conditions (*e.g.*, upon induction) but not to be substantially expressed in undesired cell types and/or under undesired conditions. Thus, the site and degree of expression of a particular nucleic acid sequence is regulated in a desired fashion. This is accomplished by, for example, introducing site-specific nucleotide substitutions, deletions, or insertions to create a nucleotide sequence that comprises a control element that is selectively expressed in the desired cell type and/or under desired conditions. This can be accomplished entirely by changing nucleotides that are already present in the target locus, or by incorporating into the target locus an exogenous DNA that includes a sequence that functions as all or part of a control element, or by a combination of these modifications.

For example, one can use the claimed methods to introduce or disrupt a response element, which is a cis-acting nucleic acid sequence that interacts with a trans-activating or trans-repressing compound (usually a protein or a protein complexed with another material) to respectively stimulate or suppress transcription. Response elements that can be introduced or eliminated using the claimed methods include cell-selective response elements, hormone receptor response elements, carbohydrate response elements, antibiotic response elements, and the like. A cell-selective response element is capable of being

activated by a trans-activating regulatory element that is selectively produced in the cell type(s) of interest. The choice of cell-selective response element used in the claimed methods depends upon whether the cell in which induction or repression of expression is desired produces the trans-activator that acts on the response element. For example, selective expression of a gene in pancreatic acinar cells, lens tissue, B cells, liver cells, and HIV-infected cells can be achieved by using the claimed methods to introduce an elastase I enhancer, a gamma crystallin gene response elements, an immunoglobulin heavy and/or light chain enhancer, a liver enhancer such as an I-1-antitrypsin or serum albumin enhancer, a chorionic gonadotropin I-chain or ϑ-chain enhancer, an interleukin-2 (IL-2) enhancer, an IL-2 receptor enhancer, or a human immunodeficiency virus (HIV) response element such as the TAR site, respectively.

Hormone receptor response elements, which can be activated or repressed when a hormone, or a functional equivalent thereof, interacts with a cellular receptor for that hormone, can be introduced into a desired location using the claimed methods. The hormone-receptor complex is internalized by the cell, where it selectively interacts with the appropriate hormone receptor response element (either directly or indirectly), thereby activating or repressing expression of genes operatively linked to the element. To obtain hormone-responsive induction or repression of expression, the claimed methods are used to create a hormone response element upstream of a gene to be regulated. Expression of the gene will be regulated by the hormone in those cells that express receptors for the given hormone.

An antibiotic response element is regulated by the presence or absence of an antibiotic. For example, a tetracycline response element is responsive to tetracycline. Similarly, a carbohydrate response element is regulated by the presence or absence of certain carbohydrates or analogs thereof. Other response elements, as well as promoters, enhancers, and other regulatory regions, are well known to those of skill in the art. These can also be created or destroyed by use of the claimed methods.

The claimed methods can also be used to modify nucleic acid sequences that are involved in other cellular processes such as DNA replication (*see, e.g.*,, Komberg and Baker, *DNA Replication*, 2nd Ed., WH Freeman & Co., 1991), as well as matrix attached regions (*see, e.g.*, Bode *et al.* (1996) *Crit. Rev. Eukaryot. Gene. Expr.* 6: 115-38; Boulikas,

(1993) *J. Cell. Biochem.* 52: 14-22), chromatin recombination hotspots (*see, e.g.*, Smith (1994) *Experientia* 50: 234-41), and the like.

Through their use of parvoviral vectors to deliver the recombinant viral genome to a cell, the claimed methods result in desired specific genetic modification events occurring at a much higher frequency than previously possible with other methods of site-specific modification of DNA in vertebrate cells. Desired modification frequencies of greater than 0.01% or greater are typically obtained using the claimed methods; indeed, efficiencies greater than 0.1%, and even greater than 1% can be obtained using the methods. The efficiency of genetic modification depends in part on the multiplicity of infection (MOI; defined herein in units of vector particles per cell) used for the transduction, as well as the type of cell being transduced. In a typical embodiment, a MOI of about 1 to 10^{12} is used to transduce a cell obtained from a continuous cell line; more preferably the MOI is at least about 10^4, and most preferably the MOI used in the claimed methods is at least about 10^6 vector particles per cell.

The methods are useful for introducing genetic modifications into any cells that are susceptible to transduction by the recombinant parvoviral vectors. Such cells can be obtained from many vertebrate species, including mammals, birds, reptiles, amphibians, fish, and the like. For example, cells from mammals such as human, cow, pig, goat, sheep, rodent, and the like can be modified using these methods. Cells that can be modified using the claimed methods include brain, muscle, liver, lung, bone marrow, heart, neuron, gastrointestinal, kidney, spleen, and the like. Also amenable to genetic modification using the claimed methods are germ cells, including ovum and sperm, fertilized egg cells, embryonic stem cells, and other cells that are capable of developing into an organism, or a part of an organism such as an organ.

Both primary cells (also referred to herein as "normal cells") and cells obtained from a cell line are amenable to modification using the claimed methods. Primary cells include cells that are obtained directly from an organism or that are present within an organism, and cells that are obtained from these sources and grown in culture, but are not capable of continuous (*e.g.*, many generations) growth in culture. For example, primary fibroblast cells are considered primary cells. The methods are also useful for modifying the genomes of cells obtained from continuous, or immortalized, cell lines, including, for

example, tumor cells and the like, as well as tumor cells obtained from organisms. Such cells can be modified *in vitro*, *ex vivo*, or *in vivo*.

The methods are useful for modifying the genomes of vertebrate cell organelles, as well as nuclear genomes. For example, one can use the methods of the invention to modify a target locus in the mitochondrial genome of a cell by including in the recombinant viral genome a targeting construct that, except for the desired modification or modifications, is at least substantially identical to a target locus in the mitochondrial genome.

A. Preparing Vectors

The practice of this invention involves the construction of parvoviruses having recombinant viral genomes and the packaging of these viral genomes into viral particles. Methods for achieving these ends are known in the art. A wide variety of cloning and *in vitro* amplification methods suitable for the construction of recombinant viral genomes are well-known to persons of skill. Examples of these techniques and instructions sufficient to direct persons of skill through many cloning exercises are found in Berger and Kimmel, *Guide to Molecular Cloning Techniques, Methods in Enzymology* 152 Academic Press, Inc., San Diego, CA (Berger); Sambrook *et al.* (1989) *Molecular Cloning - A Laboratory Manual* (2nd ed.) Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor Press, NY, (Sambrook); *Current Protocols in Molecular Biology*, F.M. Ausubel *et al.*, eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1994 Supplement) (Ausubel); Cashion *et al.*, U.S. patent number 5,017,478; and Carr, European Patent No. 0,246,864.

Examples of techniques sufficient to direct persons of skill through *in vitro* amplification methods are found in Berger, Sambrook, and Ausubel, as well as Mullis *et al.* (1987) U.S. Patent No. 4,683,202; *PCR Protocols A Guide to Methods and Applications* (Innis *et al.* eds) Academic Press Inc. San Diego, CA (1990) (Innis); Arnheim & Levinson (October 1, 1990) *C&EN* 36-47; *The Journal Of NIH Research* (1991) 3: 81-94; Kwoh *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86: 1173; Guatelli *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87: 1874; Lomell *et al.* (1989) *J. Clin. Chem* 35: 1826; Landegren *et al.* (1988) *Science* 241: 1077-1080; Van Brunt (1990) *Biotechnology* 8: 291-294; Wu and Wallace (1989) *Gene* 4, 560; and Barringer *et al.* (1990) *Gene* 89: 117. Oligonucleotide synthesis, useful in

cloning or amplifying nucleic acids, is typically carried out on commercially available solid phase oligonucleotide synthesis machines (Needham-VanDevanter *et al.* (1984) *Nucleic Acids Res.* 12:6159-6168) or chemically synthesized using the solid phase phosphoramidite triester method described by Beaucage *et. al.* ((1981) *Tetrahedron Letts.* 22 (20): 1859-1862.

Typically, the recombinant viral genomes are initially constructed as plasmids using standard cloning techniques. The targeting constructs are inserted into the viral genomes, which include at least one of the two inverted terminal repeats or their functional equivalent, and viral sequences necessary for replication and packaging of the recombinant viral genome into virions. The recombinant viral genomes are grown as a plasmid and packaged into virions by standard methods. *See, e.g.*, Muzyczka, *supra.*, Russell *et al.* (1994) *Proc. Nat'l. Acad. Sci. USA* 91: 8915-8919, Alexander *et al.* (1996) *Human Gene Ther.* 7: 841-850; Koeberl *et al.* (1997) *Proc. Nat'l. Acad. Sci. USA* 94: 1426-1431; Samulski *et al.* (1989) *J. Virol.* 63: 3822-3828; Tratschin *et al.* (1985) *Mol. Cell. Biol.* 5: 3251-3260; and Hermonat and Muzyczka (1984) *Proc. Nat'l. Acad. Sci. USA* 81: 6466-6470.

Methods of transfecting and expressing genes in vertebrate cells are known in the art. Transducing cells with viral vectors can involve, for example, incubating vectors with cells within the viral host range under conditions and concentrations necessary to cause transduction. *See, e.g., Methods in Enzymology*, vol. 185, Academic Press, Inc., San Diego, CA (D.V. Goeddel, ed.) (1990) or M. Krieger, *Gene Transfer and Expression -- A Laboratory Manual*, Stockton Press, New York, NY; and Muzyczka (1992) *Curr. Top. Microbiol. Immunol.* 158: 97-129, and references cited in each. The culture of cells, including cell lines and cultured cells from tissue samples is well known in the art. Freshney (*Culture of Animal Cells, a Manual of Basic Technique, third edition* Wiley-Liss, New York (1994)) provides a general guide to the culture of cells.

B. Identification of Cells having Genetic Modifications

Because of the high frequencies with which specific genetic modifications occur using the claimed methods, selection or screening for individual cells that include the desired modification is not necessary for many uses. Where it is desirable to identify cells that have incorporated a desired genetic modification, one can use techniques that are well known to those of skill in the art. For example, PCR and related methods (such as ligase chain reaction) are routinely used to detect specific changes in nucleic acids (*see*, Innis,

supra, for a general description of PCR techniques). Hybridization analysis under conditions of appropriate stringency are also suitable for detecting specific genetic modifications. Many assay formats are appropriate, including those reviewed in Tijssen (1993) *Laboratory Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes, Parts I and II*, Elsevier, New York; and Choo (ed) (1994) *Methods In Molecular Biology* Volume 33- *In Situ Hybridization Protocols*, Humana Press Inc., New Jersey (see also, other books in the *Methods in Molecular Biology* series). A variety of automated solid-phase detection techniques are also appropriate. For instance, very large scale immobilized polymer arrays (VLSIPS™) are used for the detection of specific mutations in nucleic acids. See, Tijssen (*supra*), Fodor *et al.* (1991) *Science*, 251: 767- 777 and Sheldon *et al.* (1993) *Clinical Chemistry* 39(4): 718-719.

These methods can be used to detect the specific genetic modifications themselves, or can be used to detect changes that result from the modification. For example, one can use hybridization or other methods to detect the presence or absence of a particular mRNA in a cell that has a modification in the promoter region.

One can also detect changes in the phenotype of the cells by other methods. For example, where a genetic modification results in a polypeptide being expressed in modified cells under conditions that an unmodified cell would not express the polypeptide, or vice versa, antibodies against the polypeptide can be used to detect expression. When the modified cells are in a vertebrate, the antibodies can be used to detect the presence or absence of the protein in the bloodstream or other tissue, for example. Where the genetic modification changes the structure of a polypeptide, one can obtain an antibody that recognizes the unmodified polypeptide but not the modified version, or vice versa. Methods of producing polyclonal and monoclonal antibodies are known to those of skill in the art, and many antibodies are available. *See, e.g.*, Coligan (1991) *Current Protocols in Immunology* Wiley/Greene, NY; and Harlow and Lane (1989) *Antibodies: A Laboratory Manual,* Cold Spring Harbor Press, NY; Stites *et al.* (eds.) *Basic and Clinical Immunology* (4th ed.) Lange Medical Publications, Los Altos, CA, and references cited therein; Goding (1986) *Monoclonal Antibodies: Principles and Practice* (2d ed.) Academic Press, New York, NY; and Kohler and Milstein (1975) Nature 256: 495-497. Other suitable techniques for antibody preparation include selection of libraries of recombinant antibodies in phage or

similar vectors. *See*, Huse *et al.* (1989) *Science* 246: 1275-1281 and Ward *et al.* (1989) *Nature* 341: 544-546. Vaughan *et al.* (1996) *Nature Biotechnology*, 14: 309-314 describe human antibodies with subnanomolar affinities isolated from a large non-immunized phage display library. Chhabinath *et al.* describe a knowledge-based automated approach for antibody structure modeling ((1996) *Nature Biotechnology* 14: 323-328). Specific monoclonal and polyclonal antibodies and antisera will usually bind to their corresponding antigen with a K_D of at least about 0.1 mM, more usually at least about 1 TM, preferably at least about 0.1 TM or better, and most typically and preferably, 0.01 TM or better. One can also detect the enzymatic activity (or loss thereof) of the modified enzyme.

Genetically modified cells can also be identified by use of a selectable or screenable marker that is incorporated into the cellular genome. A selectable marker can be a gene that codes for a protein necessary for the survival or growth of the cell, so only those host cells that contain the marker are capable of growth under selective conditions. For example, where the claimed methods are used to introduce a genetic modification that places a gene that is required for cell growth under the control of an inducible promoter, cells that have incorporated the desired modification can be selected by growing the cells under selective conditions that also induce expression of the gene. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxic substances, *e.g.*, gancyclovir, neomycin, hygromycin, G418, methotrexate, etc.; (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media. The choice of the proper selectable marker will depend on the host cell, and appropriate markers for different hosts are well known in the art. A screenable marker is a gene that codes for a protein whose activity is easily detected, allowing cells expressing such a marker to be readily identified. Such markers include, for example, ϑ-galactosidase, ϑ-glucuronidase, and luciferase.

C. *In Vitro* Uses

The claimed methods are useful for constructing cells and cell lines that are useful for numerous purposes. Genetically modified cells can be used to produce a desired gene product at a greater level than otherwise produced by the cells, or a gene product that is modified from that otherwise produced. For example, one can modify a nonhuman cell gene that encodes a desired protein so that the amino acid sequence of the encoded protein corresponds to that of the human form of the protein. Or the amino acid sequence can be

changed to make the protein more active, more stable, have a longer therapeutic half-life, have a different glycosylation pattern, and the like. The methods can be used to introduce a signal sequence at the amino terminus of a protein, which can facilitate purification of the protein by causing the cell to secrete a protein that is normally not secreted.

As another example, one can use the claimed methods to modify cells to make them express a polypeptide that, for example, is involved in degradation of a toxic compound. If desired, expression can be made inducible by the presence of the toxic compound. Such cells can be used for bioremediation of toxic waste streams and for cleanup of contaminated sites.

Cells that have been modified using the claimed methods are also useful for studying the effect of particular mutations. For example, one can disrupt expression of a particular gene and determine the effect of that mutation on growth and/or development of the cell, and the interactions of the cell with other cells. Genes suspected of involvement in disease, such as tumorigenesis and other diseases, can be disrupted to determine the effect on disease development. Alternatively, expression of disease-related genes can be turned on or elevated and the effect evaluated.

Cells that are modified to express a particular gene under given conditions can be used to screen for compounds that are capable of inhibiting the expression of the gene. For instance, a cell can be modified to place a gene required for cell growth under the control of an inducible promoter. Test compounds are added to the growth medium along with the moiety that induces expression of the gene; cells in the presence of a test compound that inhibits the interaction between the inducing moiety and the inducible promoter will not grow. Thus, these cells provide a simple screening system for compounds that modulate gene expression.

Many other uses for the claimed methods of introducing genetic mutations will be apparent to those of skill in the art.

D. Construction of Transgenic and Chimeric Animals

The invention also provides methods producing transgenic and chimeric animals, and transgenic and chimeric animals that are produced using these methods. A "chimeric animal" includes some cells that contain one or more genomic modifications introduced using the methods and other cells that do not contain the modification. A

"transgenic animal," in contrast, is made up of cells that have all incorporated the specific modification or modifications. While a transgenic animal is capable of transmitting the modified target locus to its progeny, the ability of a chimeric animal to transmit the modification depends upon whether the modified target locus is present in the animal's germ cells. The modifications can include, for example, insertions, deletions, or substitutions of one or more nucleotides.

The claimed methods are useful for producing transgenic and chimeric animals of most vertebrate species. Such species include, but are not limited to, nonhuman mammals, including rodents such as mice and rats, rabbits, ovines such as sheep and goats, porcines such as pigs, and bovines such as cattle and buffalo. Methods of obtaining transgenic animals are described in, for example, Puhler, A., Ed., *Genetic Engineering of Animals*, VCH Publ., 1993; Murphy and Carter, Eds., *Transgenesis Techniques : Principles and Protocols* (*Methods in Molecular Biology*, Vol. 18), 1993; and Pinkert, CA, Ed., *Transgenic Animal Technology : A Laboratory Handbook*, Academic Press, 1994. Transgenic fish having specific genetic modifications can also be made using the claimed methods. *See, e.g.*, Iyengar *et al.* (1996) *Transgenic Res.* 5: 147-166 for general methods of making transgenic fish.

One method of obtaining a transgenic or chimeric animal having specific modifications in its genome is to contact fertilized oocytes with a parvoviral vector that includes a targeting construct that has the desired modifications. For some animals, such as mice fertilization is performed *in vivo* and fertilized ova are surgically removed. In other animals, particularly bovines, it is preferably to remove ova from live or slaughterhouse animals and fertilize the ova *in vitro*. *See* DeBoer *et al.*, WO 91/08216. *In vitro* fertilization permits the modifications to be introduced into substantially synchronous cells. Fertilized oocytes are then cultured *in vitro* until a pre-implantation embryo is obtained containing about 16-150 cells. The 16-32 cell stage of an embryo is described as a morula. Pre-implantation embryos containing more than 32 cells are termed blastocysts. These embryos show the development of a blastocoel cavity, typically at the 64 cell stage. If desired, the presence of a desired modification in the embryo cells can be detected by methods known to those of skill in the art. Methods for culturing fertilized oocytes to the pre-implantation stage are described by Gordon *et al.* (1984) *Methods Enzymol.* 101: 414; Hogan *et al.*

Manipulation of the Mouse Embryo: A Laboratory Manual, C.S.H.L. N.Y. (1986) (mouse embryo); Hammer *et al.* (1985) *Nature* 315: 680 (rabbit and porcine embryos); Gandolfi *et al.* (1987) *J. Reprod. Fert.* 81: 23-28; Rexroad *et al.* (1988) *J. Anim. Sci.* 66: 947-953 (ovine embryos) and Eyestone *et al.* (1989) *J. Reprod. Fert.* 85: 715-720; Camous *et al.* (1984) *J. Reprod. Fert.* 72: 779-785; and Heyman *et al.* (1987) *Theriogenology* 27: 5968 (bovine embryos). Sometimes pre-implantation embryos are stored frozen for a period pending implantation. Pre-implantation embryos are transferred to an appropriate female resulting in the birth of a transgenic or chimeric animal depending upon the stage of development when the transgene is integrated. Chimeric mammals can be bred to form true germline transgenic animals.

Alternatively, the parvoviral vectors can be used to introduce specific genetic modifications into embryonic stem cells (ES). These cells are obtained from preimplantation embryos cultured *in vitro*. *See, e.g.*, Hooper, ML, *Embryonal Stem Cells : Introducing Planned Changes into the Animal Germline* (Modern Genetics, v. 1), Int'l. Pub. Distrib., Inc., 1993; Bradley *et al.* (1984) *Nature* 309, 255-258. Transformed ES cells are combined with blastocysts from a nonhuman animal. The ES cells colonize the embryo and in some embryos form the germ line of the resulting chimeric animal. *See* Jaenisch, *Science*, 240: 1468-1474 (1988). Alternatively, ES cells or somatic cells that can reconstitute an organism ("somatic repopulating cells") can be used as a source of nuclei for transplantation into an enucleated fertilized oocyte giving rise to a transgenic mammal. *See, e.g.*, Wilmut *et al.* (1997) *Nature* 385: 810-813.

For production of transgenic animals containing two or more modified target loci, parvoviral vectors containing two targeting constructs can be used, or more preferably two different parvoviral vectors, each containing a different targeting construct, are introduced simultaneously using the same procedure as for modifying a single target locus. Alternatively, each modification can be initially introduced into separate animals and then combined into the same genome by breeding the animals. Or a first transgenic animal is produced that includes one of the desired modifications, after which the second modification is introduced into fertilized ova or embryonic stem cells from that animal.

E. *Ex Vivo* Applications

The methods of the invention are useful for *ex vivo* therapy, in which cells are removed from an organism, genetically modified using the claimed methods, and reintroduced into an organism. In some applications genetically modified cultured cell lines will be introduced into an organism. The genetically modified cells can be introduced into the same organism from which the cells were originally obtained, or can be introduced into a different organism of the same or a different species. *Ex vivo* therapy is useful, for example, in treating genetic diseases such as hemophilia and certain types of thalassemia, as well as other diseases that are characterized by a defect in a cell that can be removed from the animal, modified using the claimed methods, and reintroduced into the organism. The cells can be, for example, hematopoietic stem cells, which are derived from bone marrow or fetal cord blood, T-lymphocytes, B-lymphocytes, monocytes, liver cells, muscle cells, fibroblasts, stromal cells, skin cells, or stem cells. The cells can be cultured from a patient, or can be those stored in a cell bank (*e.g.*, a blood bank). These methods are useful for treating humans, and also for veterinary purposes.

The transduced cells are administered to the animal or patient at a rate determined by the LD_{50} of transduced cell type, and the side-effects of cell type at various concentrations, as applied to the mass and overall health of the patient. Administration can be accomplished via single or divided doses.

Animal models and clinical protocols for *ex vivo* gene therapy have been established for hematopoietic cells (Blaese *et al.* (1995) *Science* 270: 475-480; Kohn *et al.* (1995) *Nature Med.* 1: 1017-1023), liver cells (Grossman *et al.* (1994) *Nature Genet.* 6: 335-341), muscle cells (Bonham *et al.* (1996) *Human Gene Ther.* 7: 1423-1429), skin cells (Choate *et al.* (1996) *Nature Med.* 2: 1263-1267) and fibroblasts (Palmer *et al.* (1989) *Blood* 73: 438-445).

F. *In Vivo* Therapy

The claimed methods are useful for correcting genetic defects *in vivo*. Muscular dystrophy is just one example of a genetic disease that is often the result of one or a few mutations that result in an abnormal polypeptide being expressed that is unable to carry out its function properly. The precise mutations for many variants of these and other genetic diseases are known to those of skill in the art, as are methods for identifying

undesirable genetic mutations. Examples include, but are not limited to, Charcot-Marie-tooth disease, Coffin-Lowry syndrome, cystic fibrosis, fragile x syndrome, hemophilia, hereditary thrombotic predisposition (Factor V mutation) Huntington's disease, medium-chain acyl-coemzyme a dehydrogenase deficiency (mcad), myotonic dystrophy, neurofibromatosis (nf1), sickle cell disease and globin chain variations, spinal muscular atrophy, spincocerebellar ataxia, I and 9 thalassemia, von Hippel-Lindau disease, and the like. Genetic diseases are reviewed in, for example, Shaw, DJ (Ed.), *Molecular Genetics of Human Inherited Disease*, John Wiley & Sons, 1995; Davies and Read, *Molecular Basis of Inherited Disease*, 2nd Edition, IRL Press, 1992. Human genetic diseases are treatable using the claimed methods, as are those of other vertebrates.

The parvoviruses containing recombinant viral genomes can be administered directly to the organism for transduction of cells *in vivo*. Administration can be by any of the routes normally used for introducing virus into ultimate contact with blood or tissue cells. The viral vectors used in the present inventive method are administered in any suitable manner, preferably with pharmaceutically acceptable carriers. Suitable methods of administering such viral vectors in the context of the present invention to a patient are available, and, although more than one route can be used to administer a particular viral vector, a particular route can often provide a more immediate and more effective reaction than another route.

Pharmaceutically acceptable carriers are determined in part by the particular viral vector being administered, as well as by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable formulations of the pharmaceutical compositions of the present invention.

Formulations suitable for oral administration can consist of (a) liquid solutions, such as an effective amount of the vector dissolved in diluents, such as water, saline or PEG 400; (b) capsules, sachets or tablets, each containing a predetermined amount of the active ingredient, as liquids, solids, granules or gelatin; (c) suspensions in an appropriate liquid; and (d) suitable emulsions. Tablet forms can include one or more of lactose, sucrose, mannitol, sorbitol, calcium phosphates, corn starch, potato starch, tragacanth, microcrystalline cellulose, acacia, gelatin, colloidal silicon dioxide, croscarmellose sodium, talc, magnesium stearate, stearic acid, and other excipients,

colorants, fillers, binders, diluents, buffering agents, moistening agents, preservatives, flavoring agents, dyes, disintegrating agents, and pharmaceutically compatible carriers. Lozenge forms can comprise the active ingredient in a flavor, usually sucrose and acacia or tragacanth, as well as pastilles comprising the active ingredient in an inert base, such as gelatin and glycerin or sucrose and acacia emulsions, gels, and the like containing, in addition to the viral vector, carriers known in the art.

The viral vector, alone or in combination with other suitable components, can be made into aerosol formulations to be administered via inhalation. Because the bronchial passageways are the usual route of choice for certain viruses, corresponding vectors are appropriately administered by this method. Aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like.

Suitable formulations for rectal administration include, for example, suppositories, which consist of the active viral vector with a suppository base. Suitable suppository bases include natural or synthetic triglycerides or paraffin hydrocarbons. In addition, it is also possible to use gelatin rectal capsules which consist of a combination of the viral vector with a base, including, for example, liquid triglycercides, polyethylene glycols, and paraffin hydrocarbons.

Formulations suitable for parenteral administration, such as, for example, by intraarticular (in the joints), intravenous, intramuscular, intradermal, intraperitoneal, intrathecal (in the cerebrospinal fluid), and subcutaneous routes, include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. The formulations can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials, and in some embodiments, can be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, water, for injections, immediately prior to use. Extemporaneous injection solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described.

The dose administered to a patient, in the context of the present invention should be sufficient to effect a beneficial therapeutic response in the patient over time. The dose will be determined by the efficacy of the particular viral vector employed and the condition of the patient or animal, as well as the body weight or surface area of the patient to be treated. The size of the dose also will be determined by the existence, nature, and extent of any adverse side-effects that accompany the administration of a particular vector or transduced cell type in a particular patient or animal.

In determining the effective amount of the viral vector to be administered in the treatment or prophylaxis of a particular disease, the physician or veterinarian needs to evaluate circulating plasma levels, vector toxicities, and progression of the disease.

In the practice of this invention, the parvoviral vectors can be administered, for example, by aerosolization and inhalation, intravenous infusion, orally, topically, intramuscularly, intraperitoneally, intravesically or intrathecally. The preferred method of administration will often be intravenous or by inhalation, but the parvovirus can be applied in a suitable vehicle for the local and topical treatment of virally-mediated conditions.

For administration, parvoviruses and transduced cell types of the present invention can be administered at a rate determined by the LD_{50} of the parvovirus, and the side-effects of the parvoviral vector or cell type at various concentrations, as applied to the mass and overall health of the patient. Administration can be accomplished via single or divided doses.

Protocols for *in vivo* gene therapy using adeno-associated viral vectors have been described for the brain (Alexander *et al.* (1996) *Human Gene Ther.* 7: 841-850), liver (Koeberl *et al.* (1997) *Proc. Nat'l. Acad. Sci. USA* 94: 1426-1431), lung (Flotte *et al.* (1993) *Proc. Nat'l. Acad. Sci. USA* 90: 10613-10617), and muscle (Xiao *et al.* (1996) *J. Virol.* 70: 8098-8108). These methods can be adapted to other target organs by those of skill in the art.

EXAMPLES

The following examples are offered to illustrate, but not to limit the present invention.

Experimental procedures

A. Cell Culture

HeLa (Scherer *et al.* (1953) *J. Exp. Med.* 97: 695-709), HT-1080 (Rasheed *et al.* (1974) *Cancer* 33: 1027-33), and 293 (Graham *et al.* (1977) *J. Gen. Virol.* 36, 59-74) cells were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% heat-inactivated (56°C for 30 minutes) fetal bovine serum (HyClone, Logan, UT), 1.25 Tg/ml amphotericin, 100 U/ml of penicillin, and 100 Tg/ml of streptomycin at 37°C in a 10% CO_2 atmosphere. HT-1080 cells were maintained in HAT medium (DMEM containing 13.61 Tg/ml hypoxanthine, 0.176 Tg/ml aminopterin and 3.875 Tg/ml thymidine prior to their use in transduction experiments to minimize the number of $HPRT^-$ cells in the population.

HSNO39 cells were created by cotransfection of HeLa cells with a *Bam*H I fragment containing the SV40 replication origin and promoter, mutant *neo* gene and p15A origin (the same fragment present in pASNO39; see Figure 1A and below), and a *Bst*YI fragment of pLHL containing the Moloney murine leukemia virus long terminal repeat promoter and hygromycin resistance gene. Transfected cells were selected for by growth in 0.2 mg/ml hygromycin (Calbiochem, San Diego, CA). HSNO39 cells were derived from a single hygromycin-resistant colony and shown by Southern analysis to contain 3 copies of the *neo* gene per cell. HSNO39 cells were cultured in medium containing 0.2 mg/ml hygromycin prior to their use in transduction experiments.

B. Plasmids

The plasmids pAAV/Ad (Samulski *et al.* (1989) *J. Virol.* 63: 3822-8), pACYC184 (Chang *et al.* (1978) *J. Bacteriol.* 134: 1141-56), pBluescript (Stratagene, La Jolla, CA), psub201 (Samulski *et al.* (1987) *J. Virol.* 61: 3096-101), pSV2neo (Southern and Berg (1982) *J. Mol. Appl. Genet.* 1: 327-41) and pTR (Ryan *et al.* (1996) *J. Virol.* 70: 1542-53) have been described. pLHL was a gift from A. D. Miller (Fred Hutchinson Cancer Research Center, Seattle, WA). pRepCap2 contains the *Xba*I fragment of psub201 containing the AAV2 *rep* and *cap* genes in the *Xba*I site of pBluescript. pASNori2 was constructed by inserting a *Bam*HI - *Esp*3I *neo* fragment of pSV2neo containing a *Ssp*I - *Bst*1107I origin fragment from pACYC184 in the *Bst*BI site (end-filled with Klenow fragment of DNA Polymerase I) downstream of the *neo* gene into the *Bgl*II sites of the AAV

vector backbone of pTR after attaching *Bam*HI linkers to the pSV2neo *Esp*3I site. This same *neo* fragment was also used to construct the HSNO39 cell line. pASNO39 is identical to pASNori2 except for a *Sal*I linker (5-CGGTCGACCG) in the end-filled *Eag*I site. pASNO648 is identical to pASNori2 except for an end-filled and religated *Csp*I site. pAHPe2/3 contains bp 14,057-17,809 of the human HPRT locus (GenBank HUMHPRTB) in the *Bgl*II site of pTR as determined by DNA sequencing. pAHPe2/3X is identical to pAHPe2/3 except for an end-filled and religated *Xho*I site. Both orientations of HPRT sequences relative to the pTR backbone were obtained and no differences were noted in the homologous recombination rates of the corresponding vectors. Human HPRT sequences were from the HuΣ3 lambda phage previously described (Patel *et al.* (1986) *Mol. Cell. Biol.* 6: 393-403).

C. Vector Production

AAV vector stocks were prepared as follows. 293 cells were plated at a density of 4 x 10^6 cells/dish in 24 dishes (10 cm). The next day each dish was infected with 5.6 x 10^7 plaque-forming units of adenovirus type 5 (ATCC VR-5) and two hours later cotransfected with 4 Tg of vector plasmid and 16 Tg of helper plasmid by the calcium phosphate method (Sambrook, *supra.*). After 3 days the cells and medium were harvested, freeze-thawed 3 times, clarified by centrifugation at 5800 x g (5500 rpm) in a Sorvall HS4 rotor for 30 min. at 4°C, digested with 68 units/ml of micrococcal nuclease (Pharmacia, Piscataway, NJ) at 37°C for 1 hour, treated with 50 ng/ml of trypsin at 37°C for 30 min., and centrifuged through 40% sucrose in phosphate buffered saline in a Beckman SW28 rotor at 27,000 rpm for 16 hours at 4°C. The pellets were resuspended in 8 ml of a 0.51 g/ml solution of CsCl and centrifuged in a Beckman SW41 rotor at 35,000 rpm for 20 hours at 4°C. The region of the gradient containing AAV virions was collected, dialyzed against DMEM through a 50,000 molecular weight cutoff membrane (Spectrum, Houston, TX) and concentrated by centrifugation in Centricon 100 filters (Amicon, Inc., Beverly, MA). The vector plasmids used were pASNori2 for AAV-SNori, pASNO648 for AAV-SNO648, pASNO39 for AAV-SNO39, pAHPe2/3 for AAV-HPe2/3 and pAHPe2/3X for AAV-HPe2/3X. The helper plasmids used were pAAV/Ad (Samulski *et al.* (1989) *supra.*) or pRepCap2, which produced equivalent stock titers.

The titer of each vector stock was determined by Southern blots of alkaline gels as follows. Ten Tl stock dilutions were mixed with 2 Tl of 10% SDS, heated to 100°C for 10 minutes, electrophoresed through 1.2 % alkaline agarose gels (Sambrook, *supra.*), blotted onto Hybon-N membranes (Amersham, Buckinghamshire, England) and probed for vector sequences. The amount of full-length linear vector DNA present in each sample was determined by comparison to standards present on the same gel using a Molecular Dynamics PhosphorImager 400S (Sunnyvale, CA), and the number of vector genomes per ml of stock calculated from this measurement. The same assay was used to locate vector particles on CsCl gradients by electrophoresing 10 Tl of each gradient fraction. The number of intact vector genomes per ml of stock was the value used for vector particle numbers, which were typically $>10^{11}$/ml.

D. DNA Techniques

Enzymes were obtained from New England BioLabs, (Beverly, MA) Boehringer Mannheim, (Indianapolis, IN) or Stratagene (La Jolla, CA) and reactions were performed by using the manufacturers recommended conditions. Plasmid construction, DNA purification, Southern blot analysis and bacterial culture were performed by standard procedures (Sambrook *et al.*, *supra.*). Plasmids were prepared by using Qiagen columns (Chatsworth, CA). Dye terminator cycle sequencing was carried out using the ABI PRISM sequencing kit (Perkin Elmer, Foster City, CA) and analyzed on an Applied Biosystems Inc. sequencer (Foster City, CA). Oligonucleotides were from Cruachem, Inc. (Dulles, VA).

Integrated *neo* genes were rescued from transduced HSNO39 cells by digesting high molecular weight genomic DNA calf intestinal phosphatase to prevent ligation of free ends in the sample, heat inactivated, extracted with phenol and chloroform, and precipitated with ethanol. The resuspended DNA was digested with *Bam*HI, extracted with phenol and chloroform and precipitated with ethanol. The resulting DNA fragments were resuspended, circularized with of T4 DNA ligase at 14°C overnight and transferred to *E. coli* by electroporation or high efficiency chemical transformation. Bacterial colonies were selected for by growth on kanamycin plates.

Sequencing of the bp 39 and bp 648 mutations of corrected *neo* genes recovered as bacterial plasmids was performed with primers 9606D (dATGGCTTTCTTGCCGCCA) (SEQ ID NO:1) and 9607A

(dATACGCTTGATCCGGCTAC) (SEQ ID NO:2) respectively. HPRT exon 3 sequences were amplified from high molecular weight genomic DNA by using a modification of a previously published procedure (Rossiter *et al.* (1991) "Detection of deletions and point mutations." In *PCR. A practical approach*, M. J. McPherson *et al.*, eds. (Oxford, England: IRL Press), pp. 67-83) as follows. PCR was performed on 100 ng of genomic DNA in 20 Tl reaction volume containing 2.1 picomoles of both 5 primer (dCCTTATGAAACATGAGGGCAAAGG) (SEQ ID NO:3) and 3 primer (TGTGACACAGGCAGACTGTGGATC) (SEQ ID NO:4) , 6 mM $MgSO_4$, 1.25 mM each deoxynucleoside triphosphate, and 0.4 units Vent DNA Polymerase (New England Biolabs, Beverly, MA). The reaction was carried out in a PTC-200 thermocycler (MJ Research, Watertown, MA) with denaturation at 94°C for 4.5 minutes, followed by 30 cycles of 94°C for 30 seconds, 61°C for 50 seconds and 72°C for 2 minutes, then a final polymerization at 72°C for 5 minutes. Six Tl of the product was further amplified in a 100 Tl volume under the same conditions for 20 cycles, and the PCR product was purified using a QIAquick kit (Qiagen, Chatsworth, CA) following the manufacturers protocol, and 75 ng of the purified product was used for DNA sequencing with the primer dACCTACTGTTGCCACTA (SEQ ID NO:5).

E. Transduction Assays

Standard transduction experiments were performed by plating 5 x 10^3 or 1 x 10^4 HSNO39 cells/well respectively into 96 (Nunc, Naperville, IL) or 48 (Costar, Cambridge, MA) well plates or 2 x 10^4 HT-1080 cells into 48 well plates on day 1. On day 2, the medium was changed and vector stock (prepared in DMEM) was added to the well. The MOI was calculated assuming one cell doubling since the original plating. On day 3, each well was treated with trypsin, and the cells were plated into 10 cm dishes. On day 4, the assays differed for each cell line.

For *neo* gene correction experiments, 90%, 9.5% and 0.5% of the cells from each well were plated into different dishes. On day 4, G418 (1 mg/ml active compound) was added to the 90% and 9.5% dishes and selection was continued for 10 -12 days with medium changes every 3-4 days. G418 was not added to the 0.5% dishes which served as a control for the total number of colony-forming units (CFU) from each original well. The colonies present in each dish were counted after staining with Coomassie brilliant blue G. The *neo*

gene correction rate was calculated as the number of G418-resistant CFU/ total CFU for each original well.

For *HPRT* experiments, all the cells from each well were cultured without selection for 10-14 days after being plated into 10 cm dishes on day 3, to allow for elimination of existing HPRT protein in *HPRT*⁻ cells. No significant differences were noted in *HPRT* mutation rates after 10 day or 14 day culture periods. The medium was changed every 3-4 days and when dishes became too dense the cells were treated with trypsin and dilutions were plated into new dishes. After this phenotypic expression period, 10^5, 10^4 and 10^2 cells of each culture were plated into new 10 cm dishes, and the following day 6TG (5 Tg/ml) was added to the 10^5 and 10^4 cell dishes. 6TG selection was not applied to the 10^2 cell dishes as these were used to calculate plating efficiencies. The cells were cultured for 10 additional days, stained with Coomassie brilliant blue G, and the surviving colonies were counted. The percent of 6TG- resistant CFU was determined after correcting for plating efficiencies.

Example 1: Correction of Mutant *Neo* Genes using Adeno-associated viral vectors

This Example demonstrates that vectors based on adeno-associated virus (AAV) can efficiently modify specific chromosomal target sequences in human cells.

We used the selectable neomycin phosphotransferase gene (*neo*) as a marker to study gene correction by transduction. The vectors constructed for these experiments were based on the AAV shuttle vector AAV-SNori (Figure 1A), which contains the *neo* gene under the control of both the bacterial Tn5 promoter and SV40 early promoters, and the p15A plasmid replication origin, which supports stable replication in *Escherichia coli* (Cozzarelli *et al.* (1968) *Proc. Nat'l. Acad. Sci. USA* 60: 992-999). The AAV2 terminal repeats flank these internal sequences and contain all the cis-acting sequences required for replication and packaging of the vector genome (McLaughlin *et al.* (1988) *J. Virol.* 62: 1963-1973; Samulski *et al.*, *supra*.). Mammalian cells transduced by AAV-SNori are resistant to G418, and the integrated proviruses can be recovered as bacterial plasmids expressing kanamycin resistance. Mutations were introduced into the AAV-SNori vector at bp 39 (a 14 nucleotide insertion) and bp 648 (a 3 nucleotide insertion) of the *neo* gene (bp 1 being the translation start codon), to generate the vectors AAV-SNO39 and AAV-SNO648.

Both mutations disrupt *neo* gene function, but homologous recombination between the two mutant genes can regenerate a functional gene and confer G418 resistance.

HeLa cells were used as a model human system to study homologous recombination by AAV vectors. A HeLa cell line containing integrated copies of the internal portion of the AAV-SNO39 genome (lacking the terminal repeats) was created by cotransfection of this fragment with a hygromycin selectable marker (see Experimental Procedures). Several hygromycin-resistant clones were isolated and screened for the presence the mutant *neo* gene cassette by Southern analysis. One cell line, designated HSNO39, appeared to contain 3 intact copies per cell of the *neo* cassette integrated at different locations and was chosen for further experiments.

A. Frequency of *Neo* Gene Correction

HSNO39 cells were infected with AAV-SNO648 vector stocks, treated with trypsin and plated at different dilutions on the following day, then selected in G418 two days after infection. Dilutions were also grown without selection to determine the total number of colony-forming units in the sample. Correction of the mutant chromosomal *neo* genes by incoming vector genomes was measured as the fraction of colonies resistant to G418. As shown in Table 1, approximately 0.1% of HSNO39 cells were resistant to G418 after infection with AAV-SNO648. This represents a minimal *neo* gene correction rate as some cells could contain silenced genes with inadequate expression levels. Infection of HeLa cells with AAV-SNO648 did not produce G418-resistant colonies, demonstrating that reversion of the bp 648 mutation in the vector did not occur at detectable rates. Similarly, G418-resistant colonies were not detected in uninfected HSNO39 cells or HSNO39 cells infected with AAV-SNO39, showing that reversion of the chromosomal bp 39 mutation did not occur. About 0.6% of HeLa cells were resistant to G418 after transduction with the AAV-SNori vector, which contains a functional *neo* gene and can integrate at random chromosomal locations by non-homologous recombination. Thus the *neo* gene correction rate was about 5-fold lower than the random vector integration rate of a similar vector.

Table 1. *Neo* Gene Correction

Cell Line	Vector/Plasmid	MOI	Fraction G418^R
HSNO39	none	-	$< 5.3 \times 10^{-5}$
"	"	-	$< 4.3 \times 10^{-5}$
"	"	-	$< 4.3 \times 10^{-5}$
"	"	-	$< 1.4 \times 10^{-5}$
HSNO39	AAV-SNO648	40,000	9.6×10^{-4}
"	"	40,000	6.7×10^{-4}
"	"	400,000	2.0×10^{-3}
"	"	400,000	1.4×10^{-3}
HeLa	AAV-SNO648	40,000	$< 6.0 \times 10^{-5}$
"	"	40,000	$< 5.7 \times 10^{-5}$
"	"	400,000	$< 6.8 \times 10^{-5}$
"	"	400,000	$< 6.6 \times 10^{-5}$
HSNO39	AAV-SNO39	375,000	$< 6.3 \times 10^{-5}$
"	"	1,500,000	$< 6.6 \times 10^{-5}$
HeLa	AAV-SNori	100,000	7.3×10^{-3}
"	"	100,000	5.3×10^{-3}

B. Structure of the Chromosomal *Neo* Genes

Several G418-resistant colonies obtained by infecting HSNO39 cells with AAV-SNO648 were isolated, expanded to approximately 2×10^7 cells, and analyzed by Southern blots. After digestion with *Bam*HI, genomic DNA from the parental HSNO39 cells contained 3 major *neo*-hybridizing bands of 2.7, 5.0 and >20 kb, representing the three integrated copies of the *neo* gene cassette (Figure 1B). A 2.7 kb *Bam*HI *neo* fragment was used to generate the HSNO39 line by cotransfection. A faint 8.0 kb band was also observed at less than one copy per cell, and may be due to methylation or mutation at one of the *Bam*HI sites in a subset of HSNO39 cells. Four out of eleven HSNO39/AAV-SNO648 G418-resistant clones (1, 2, 9 and 10) contained the same 3 major bands as th$^-$ parental line, with no additional fragments. The 8.0 kb band of clones 4 and 5 could represent the faint

band of the same size in the parental line. New bands were observed in 4 of the clones, suggesting that random vector integration had also occurred in a subset of cells exposed to the vector. Three clones were missing bands present in the parental line (3, 4 and 7), which can be explained by modification of a *Bam*HI site rather than rearranged *neo* cassettes, as no novel bands were observed in these clones. Homology between the vector and chromosomal *neo* cassettes extends up to the *Bam*HI site, so modification of the chromosomal sequence at this site by vector DNA could have destroyed the site. These results demonstrate that the majority of G418-resistant clones isolated contained at least one corrected *neo* gene without additional rearrangements due to vector integration.

C. Sequence of the Corrected *Neo* Genes

To assess the fidelity of the homologous recombination process we recovered several corrected *neo* genes in bacterial plasmids and sequenced the relevant regions. The *neo* gene cassette present in HSNO39 cells and the AAV-SNO648 vector can replicate and confer kanamycin resistance in *E. coli* (Figure 1A), allowing us to recover corrected *neo* genes as bacterial plasmids. Chromosomal DNA from the G418-resistant HSNO39/AAV-SNO648 clones shown in Figure 1B was digested with *Bam*HI, circularized with DNA ligase, and transferred to bacteria that were then selected for kanamycin resistance. As shown in Table 2, corrected *neo* genes were recovered as bacterial plasmids from 7/11 clones. It is possible that more persistent attempts to recover plasmids from the remaining 4 clones would also have been successful. Plasmids isolated from these bacteria were digested with *Bam*HI and only those with a unique site were considered correct. A 2.7 kb plasmid was recovered from each of the seven clones that by restriction digestion appeared to be a circularized *Bam*HI fragment identical to that used to produce the HSNO39 line, except for the absence of the bp 39 mutation. A second 20 kb plasmid was also recovered from clone 11, which appeared to correspond to the high molecular weight band observed on Southern blots. Apparently at least two of the *neo* genes present in this cell line had been corrected. The recovered plasmids contained wild type *neo* genes based on digestion with *Bsi*EI, which can identify the bp 39 and bp 648 mutations (see Figure 1A).

Table 2. Rescue of Corrected Neo Genes

Cell Line	Southern Results	Kan^R Colonies Recovered	Fraction Correct	Plasmid Sizes
HSNO39	2.7, 5.0, (8.0), >20 kb	0	-	-
Clone 1	no change	14	11/12	2.7 kb
Clone 2	no change	7	6/6	2.7 kb
Clone 3	θ2.7 kb	0	-	-
Clone 4	θ5.0kb	0	-	-
Clone 5	+5.5 kb	7	6/7	2.7kb
Clone 6	+(2.4, 5.2) kb	3	2/3	2.7 kb
Clone 7	θ2.7 kb	0	-	-
Clone 8	+18 kb	6	5/6	2.7 kb
Clone 9	no change	2	2/2	2.7 kb
Clone 10	no change	0	-	-
Clone 11	+ 6.6 kb	4	4/4	2.7, 20 kb

We sequenced the regions surrounding the bp 39 and bp 648 mutations of each recovered plasmid. More than 200 bp of sequence was obtained from each region and in all cases the sequence corresponded exactly to that of the wild-type *neo* gene. Thus the gene correction process led to an accurate deletion of the 14 nucleotide insertion present at the chromosomal bp 39 mutation, without additional genetic changes and without insertion of the bp 648 mutation present in the vector. However, because our assay required the presence of a functional *neo* gene, any additional mutations created during the recombination event that disrupted *neo* gene function would have been excluded from our analysis.

Example 2: Modification of the Human HPRT Gene by AAV Vectors.

We also studied homologous recombination by AAV vectors at the human hypoxanthine phosphoribosyltransferase locus (HPRT). The HPRT gene is frequently used to study mutation because $HPRT^-$ cells can be selected for by growth in the presence of 6-thioguanine (6TG), so mutagenesis at the single copy X-linked locus can be measured in diploid male cells. We used HT-1080 human fibrosarcoma cells to study recombination at

the HPRT gene because this cell line has a pseudodiploid male karyotype (Rasheed *et al.* (1974) *Cancer* 33: 1027-1033) and has been used previously in HPRT gene targeting experiments (Pikaart *et al.* (1992) *Mol. Cell. Biol.* 12: 5785-92; Zheng *et al.* (1991) *Proc. Nat'l. Acad. Sci. USA* 88: 8067-71).

AAV vectors containing a region of the human HPRT locus encompassing exons 2 and 3 were used to introduce a specific mutation into the HPRT gene of HT-1080 cells (Figure 2A). The AAV-HPe2/3 vector contains wild type genomic sequence, while the AAV-HPe2/3X vector contains a 4 nucleotide insertion in exon 3, which causes a frameshift in the HPRT coding sequence. HT-1080 cells were infected with both vectors and selected for 6TG resistance after culturing the cells for a period without selection to allow for elimination of existing HPRT protein (see Experimental Procedures). As shown in Figure 3, about 1/2000 HT-1080 cells infected with the mutant AAV-HPe2/3X vector were 6TG-resistant. This represents the minimum HPRT gene modification frequency, as HT-1080 cells are not uniformly diploid and could contain additional X chromosomes (Rasheed *et al.*, *supra.*). The AAV-HPe2/3X vector targeting frequency was about 30 fold above the background mutation rate. Infection with the wild-type vector did not raise the HPRT mutation rate above background levels.

Southern analysis of several 6TG-resistant clones isolated after infection with AAV-HPe2/3X confirmed that the vector mutation had been introduced into the chromosomal HPRT locus. Figure 2B shows the results of digestion with *Hin*dIII, which cuts outside of vector sequences and produces a 6.8 kb chromosomal fragment containing exons 2 and 3 in HT-1080 cells. This band was unaltered in all of the clones analyzed, demonstrating the absence of major rearrangements in this region. Five lines contained additional bands, presumably due to random vector integration. Further digestion with *Pvu*I showed that 10/13 clones contained the 2.2 kb band expected from transfer of the vector *Pvu*I site insertion mutation to the chromosome (Figure 2C). To date we have analyzed 24 independent 6TG-resistant HT-1080 clones infected with AAV-HPe2/3X, 18 of which had the expected *Pvu*I site insertion in exon 3 as determined by Southern analysis. We used the polymerase chain reaction (PCR) to amplify exon 3 from the genomic DNA of 6 of these clones and sequenced the PCR products (*see* Experimental Procedures). At least 330 bp of unambiguous sequence was obtained from each clone, including all of exon 3. In all cases

the entire sequence was identical to the published *HPRT* sequence except for the predicted 4 nucleotide insertion in exon 3. Clones without additional vector integration events were sequenced to avoid amplification of unlinked vector DNA. Sequence from the parental HT-1080 cell line did not contain this insertion mutation.

Example 3: The Effects of Vector Dose on Gene Correction.

The mutant *neo* gene in HSNO39 cells was corrected with the AAV-SNO648 vector using a range of infection multiplicities. Figure 4 shows the results of several experiments plotted as infecting vector genomes per cell versus the percent of cells with corrected *neo* genes. The gene correction rate increased from about 0.001 to greater than 0.1 percent with increasing vector doses of 40 to 2 x 10^6 vector particles per cell. These results suggest that the gene correction reaction is limited by the number of vector molecules entering the cell.

Example 4: Comparison of Transduction and Transfection Homologous Recombination Rates.

We compared *neo* gene correction rates in HSNO39 cells transduced with AAV-SNO648 vector stocks or transfected with the plasmid pASNO648, which contains the entire AAV-SNO648 genome (Figure 5). The transduction rate was at least 400 times that obtained by transfection. Further transfection experiments using the pASNO39 plasmid, which contains the same mutation as the HSNO39 cell line, gave similar results to pASNO648, suggesting that the gene correction rate of pASNO648 was actually due to reversion rather than homologous pairing. Thus the homologous pairing rate by transduction could have been much more than 400 times that obtained by transfection. One potential explanation for these differences is that plasmid uptake occurs in only a small proportion of transfected cells, while vector genomes presumably enter every cell. The stable transfection efficiency of HSNO39 cells was approximately 7% as determined by transfections with pASNori2, which is identical to pASNO648 except it contains a functional *neo* gene. Presumably, an even higher percentage of cells were transiently transfected. Even after making the conservative assumptions that 7% of transfected cells contained functional plasmid molecules, and that all the G418-resistant colonies obtained by transfecting pASNO648 were due to homologous pairing, the gene correction rate is still 30 fold higher

in transduced cells than that observed in the subpopulation of cells that incorporated plasmid DNA (1.7×10^{-3} vs 5.7×10^{-5}).

Example 5: Modification of HPRT Genes in Normal Human Fibroblasts

Standard transduction experiments were performed by plating 5×10^4 normal human fibroblasts per well into 24 well plates. On day two, the medium was changed and vector stock (AAV-HPe2/3 or AAV-Hpe2/3X, prepared in DMEM) was added to the well. On day three, each well was treated with trypsin and the cells were plated into 10 cm dishes. On day four, all of the cells from each well were cultured without selection for 12-14 days to allow for elimination of existing HPRT protein in *HPRT* cells. The medium was changed every 3-4 days and when cells became too dense the cells were treated with trypsin and dilutions were plated into new dishes. After this phenotypic expression period, 10^5, 10^4, and 10^2 cells of each culture were plated into new 10 cm dishes, and the following day 6TG (10 Tg/ml) was added to the 10^5 and 10^4 cell dishes. 6TG selection was not applied to the 10^2 cell dishes, as these were used to calculate plating efficiencies. The cells were cultured for ten additional days, stained with Coomassie brilliant blue G, and the surviving colonies were counted. The percentage of 6TG-resistant colony-forming units was determined after correcting for plating efficiencies. Four different normal fibroblast lines were studied: MHF1, MHF2 and MHF3 were from normal males, and FHF1 was from a normal female.

As shown in Figure 6, modification of the HPRT gene was proportional to the number of infecting viral genomes per cell. Modifications could be introduced into the *HPRT* genes of all four fibroblast lines (Figure 7).

Summary of Examples

These Examples demonstrates that vectors based on adeno-associated virus (AAV) efficiently and specifically modify vertebrate chromosomal target sequences. Both integrated neomycin phosphotransferase genes and the normal, X-linked hypoxanthine phosphoribosyltransferase gene were targeted by AAV vectors. Site-specific genetic modifications could be introduced into > 0.1% of the total cell population, a significantly higher rate than could be achieved by transfection, and the modifications could be introduced into normal primary cells. The majority of modified cells contained no other detectable genetic changes, and DNA sequencing demonstrated the high fidelity of the process. These

results suggest that parvoviral vectors are useful for introducing specific genetic changes into the genomic DNA of a wide variety of vertebrate cells.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference for all purposes.

WHAT IS CLAIMED IS:

1. A method of producing a vertebrate cell having a modification at a pre-selected target locus, said method comprising:

(a) contacting the cell with a parvoviral vector having a recombinant viral genome comprising a targeting construct that comprises a DNA sequence which is substantially identical to the target locus except for the modification being introduced; and

(b) allowing the recombinant viral genome to enter the vertebrate cell by transduction, wherein homologous pairing occurs between the targeting construct and the target locus resulting in the modifications being introduced into the target locus.

2. The method of claim 1, wherein the recombinant viral genome further comprises a DNA that is exogenous to the vertebrate cell, wherein the exogenous DNA is preselected for modification of the target locus.

3. The method of claim 2, wherein the exogenous DNA comprises a selection marker that is functional in the vertebrate cell.

4. The method of claim 1, wherein the desired modification comprises one or more deletions, insertions, substitutions, or a combination thereof.

5. The method of claim 1, wherein the method further comprises introducing a modification at one or more additional preselected target loci by repeating steps (a) and (b) using a parvoviral vector having a recombinant viral genome comprising a targeting construct that comprises a DNA sequence which is substantially identical to each of the additional target loci except for the modification being introduced.

6. The method of claim 5, wherein both targeting constructs are present in a single parvoviral vector.

7. The method of claim 1, wherein the target locus comprises a DNA sequence selected from the group consisting of a transcriptional regulatory region, a splice signal, a sequence involved in DNA replication, a matrix attachment point, a chromosomal recombination hotspot, a structural gene, or a coding region for a signal sequence, and portions thereof.

8. The method of claim 7, wherein the DNA sequence comprises a structural gene and the modification results in an amino acid substitution, deletion, insertion, or a combination thereof, in a polypeptide encoded by the gene.

9. The method of claim 7, wherein the DNA sequence comprises a transcriptional regulatory region selected from the group consisting of a promoter, an enhancer, a response element, a transcription termination signal, and a locus control region.

10. The method of claim 9, wherein a gene under the control of the modified transcriptional regulatory region is expressed at a different level than that at which the gene is expressed under equivalent conditions when the gene is under the control of the unmodified transcriptional regulatory region.

11. The method of claim 10, wherein the gene under the control of the modified transcriptional regulatory region is expressed at a higher level than that at which the gene is expressed under equivalent conditions when the gene is under the control of the unmodified transcriptional regulatory region.

12. The method of claim 11, wherein the gene under the control of the modified transcriptional regulatory region is expressed under conditions that do not result in expression of the gene when under the control of the unmodified transcriptional regulatory region.

13. The method of claim 10, wherein the transcriptional regulatory region is made inducible by the modification.

14. The method of claim 10, wherein the gene under the control of the modified transcriptional regulatory region is expressed at a lower level than that at which the gene is expressed under equivalent conditions when the gene is under the control of the unmodified transcriptional regulatory region.

15. The method of claim 14, wherein a gene under the control of the transcriptional regulatory region is not expressed by a cell having the modification under

conditions that result in expression of the gene when under the control of the unmodified transcriptional regulatory region.

16. The method of claim 1, wherein the vertebrate cell is a replicating cell.

17. The method of claim 1, wherein the vertebrate cell is a mammalian cell.

18. The method of claim 17, wherein the mammalian cell is a human cell.

19. The method of claim 18, wherein the human cell is a human fibroblast cell.

20. The method of claim 1, wherein the vertebrate cell is obtained from a cell line.

21. The method of claim 1, wherein the vertebrate cell is a primary cell.

22. The method of claim 1, wherein the vertebrate cell is a transformed, immortal, or malignant cell.

23. The method of claim 1, wherein the vertebrate cell is a cell from which an organism can be reconstituted.

24. The method of claim 23, wherein the cell is selected from the group consisting of an embryonic stem cell, a sperm cell, an ovum, a fertilized ovum, and a somatic repopulating cell.

25. The method of claim 1, wherein the parvoviral vector is an adeno-associated viral vector.

26. The method of claim 1, wherein the recombinant viral genome further comprises at least one parvoviral terminal repeat segment.

27. The method of claim 1, wherein multiple cells are subjected to the transduction method and the cells are transduced at a rate of at least about 0.01%.

28. The method of claim 27, wherein the cells are transduced at a rate of at least about 0.1%.

29. A cell having a specific genetic modification introduced at a predetermined target locus by the method of claim 1.

30. The cell of claim 29, wherein the cell is present in a vertebrate.

31. A vertebrate that comprises a cell according to claim 29.

32. A method of making an animal comprising cells which have a modification of a target locus, the method comprising:

(a) contacting a cell from which an animal can be reconstituted with a parvoviral vector having a recombinant viral genome comprising a targeting construct that comprises a DNA sequence which is substantially identical to the target locus except for the modification being introduced;

(b) allowing the recombinant viral genome to enter the cell by transduction, wherein homologous pairing occurs between the targeting construct and the target locus resulting in the modifications being introduced into the target locus; and

(c) culturing and reimplanting the cell and/or progeny of the cell into a female which carries the resulting embryo to term.

33. The method according to claim 32, wherein the animal is a transgenic animal.

34. The method according to claim 32, wherein the animal is a chimeric animal.

35. A method for introducing a modification of a target locus in a cell in a vertebrate, the method comprising:

(a) transducing a cell *ex vivo* with a parvoviral vector having a recombinant viral genome comprising a targeting construct that comprises a DNA sequence that is substantially identical to the target locus except for the modification being introduced; and

(b) allowing the recombinant viral genome to enter the cell by transduction, wherein homologous pairing occurs between the targeting construct and the target locus resulting in the modifications being introduced into the target locus; and

(c) introducing the transduced cell into a vertebrate.

36. The method of claim 35, wherein the transduced cell is introduced into the same vertebrate from which the transduced cell was obtained.

37. The method of claim 35, wherein the vertebrate is a mammal.

38. The method of claim 37, wherein the mammal is a human.

39. The method of claim 35, wherein the cell is a liver cell, a muscle cell, a fibroblast cell, a stromal cell, a skin cell, a stem cell, a hematopoietic cell, a fetal cord blood cell, a T-lymphocyte, a B-lymphocyte, or a monocyte.

40. A method for introducing a modification of a target locus in a cell in a vertebrate, the method comprising administering to the vertebrate a parvoviral vector having a recombinant viral genome comprising a targeting construct that comprises a DNA sequence that is substantially identical to the target locus except for the modification being introduced, wherein homologous pairing occurs between the targeting construct and the target locus resulting in the modifications being introduced into the target locus.

41. The method of claim 40, wherein the vertebrate is a mammal.

AMENDED CLAIMS
[received by the International Bureau on 2 October 1998 (02.10.98); original claims 1-41 replaced by new claims 1-44 (6 pages)]

1. A method of producing a vertebrate cell having a modification at a preselected target locus, said method comprising introducing into a vertebrate cell a recombinant parvoviral vector that comprises:

a) a targeting construct which comprises a DNA sequence which is substantially identical to the target locus except for the modification being introduced; and

b) all or a portion of at least one parvoviral ITR or a functional equivalent;

wherein homologous pairing occurs between the targeting construct and the target locus resulting in the modification being introduced into the target locus.

2. The method of claim 1, wherein the method comprises allowing the recombinant viral genome to enter the vertebrate cell by transduction.

3. The method of claim 1, wherein the recombinant parvoviral vector further comprises a DNA that is exogenous to the vertebrate cell, wherein the exogenous DNA is preselected for modification of the target locus.

4. The method of claim 3, wherein the exogenous DNA comprises a selection marker that is functional in the vertebrate cell.

5. The method of claim 1, wherein the desired modification comprises one or more deletions, insertions, substitutions, or a combination thereof.

6. The method of claim 1, wherein the method further comprises introducing a modification at one or more additional preselected target loci by introducing into the vertebrate cell a recombinant parvoviral vector comprising a targeting construct that comprises a DNA sequence which is substantially identical to each of the additional target loci except for the modification being introduced.

7. The method of claim 6, wherein both targeting constructs are present in a single parvoviral vector.

8. The method of claim 1, wherein the target locus comprises a DNA sequence selected from the group consisting of a transcriptional regulatory region, a splice signal, a sequence involved in DNA replication, a matrix attachment point, a chromosomal recombination hotspot, a structural gene, or a coding region for a signal sequence, and portions thereof.

9. The method of claim 8, wherein the DNA sequence comprises a structural gene and the modification results in an amino acid substitution, deletion, insertion, or a combination thereof, in a polypeptide encoded by the gene.

10. The method of claim 8, wherein the DNA sequence comprises a transcriptional regulatory region selected from the group consisting of a promoter, an enhancer, a response element, a transcription termination signal, and a locus control region.

11. The method of claim 10, wherein a gene under the control of the modified transcriptional regulatory region is expressed at a different level than that at which the gene is expressed under equivalent conditions when the gene is under the control of the unmodified transcriptional regulatory region.

12. The method of claim 11, wherein the gene under the control of the modified transcriptional regulatory region is expressed at a higher level than that at which the gene is expressed under equivalent conditions when the gene is under the control of the unmodified transcriptional regulatory region.

13. The method of claim 12, wherein the gene under the control of the modified transcriptional regulatory region is expressed under conditions that do not result in expression of the gene when under the control of the unmodified transcriptional regulatory region.

14. The method of claim 11, wherein the transcriptional regulatory region is made inducible by the modification.

15. The method of claim 11, wherein the gene under the control of the modified transcriptional regulatory region is expressed at a lower level than that at which the

gene is expressed under equivalent conditions when the gene is under the control of the unmodified transcriptional regulatory region.

16. The method of claim 15, wherein a gene under the control of the transcriptional regulatory region is not expressed by a cell having the modification under conditions that result in expression of the gene when under the control of the unmodified transcriptional regulatory region.

17. The method of claim 1, wherein the vertebrate cell is a replicating cell.

18. The method of claim 1, wherein the vertebrate cell is a mammalian cell.

19. The method of claim 18, wherein the mammalian cell is a human cell.

20. The method of claim 19, wherein the human cell is a human fibroblast cell.

21. The method of claim 1, wherein the vertebrate cell is obtained from a cell line.

22. The method of claim 1, wherein the vertebrate cell is a primary cell.

23. The method of claim 1, wherein the vertebrate cell is a transformed, immortal, or malignant cell.

24. The method of claim 1, wherein the vertebrate cell is a cell from which an organism can be reconstituted.

25. The method of claim 1, wherein a nucleus is removed from the vertebrate cell and transplanted into a cell from which an organism can be reconstituted.

26. The method of claim 24, wherein the cell is selected from the group consisting of an embryonic stem cell, a sperm cell, an ovum, a fertilized ovum, and a somatic repopulating cell.

27. The method of claim 1, wherein the parvoviral vector is an adeno-associated viral vector.

28. The method of claim 1, wherein the recombinant parvoviral vector further comprises at least one parvoviral terminal repeat segment.

29. The method of claim 1, wherein multiple cells are subjected to the transduction method and the cells are transduced at a rate of at least about 0.01%.

30. The method of claim 29, wherein the cells are transduced at a rate of at least about 0.1%.

31. A cell having a specific genetic modification introduced at a predetermined target locus by the method of claim 1.

32. The cell of claim 31, wherein the cell is present in a vertebrate.

33. A vertebrate that comprises a cell according to claim 31.

34. A method of making an animal that comprises cells which have a modification of a target locus, the method comprising:

introducing into a cell from which an animal can be reconstituted a recombinant parvoviral vector that comprises:

a) a targeting construct which comprises a DNA sequence which is substantially identical to the target locus except for the modification being introduced; and

b) all or a portion of at least one parvoviral ITR or a functional equivalent;

wherein homologous pairing occurs between the targeting construct and the target locus resulting in the modification being introduced into the target locus; and

culturing and reimplanting the cell and/or progeny of the cell into a female which carries the resulting embryo to term.

35. A method of making an animal that comprises cells which have a modification of a target locus, the method comprising:

introducing into a vertebrate cell a recombinant parvoviral vector that comprises:

c) a targeting construct which comprises a DNA sequence which is substantially identical to the target locus except for the modification being introduced; and

d) all or a portion of at least one parvoviral ITR or a functional equivalent;

wherein homologous pairing occurs between the targeting construct and the target locus resulting in the modification being introduced into the target locus;

introducing a nucleus from the cell into a second cell from which an animal can be reconstituted; and

culturing and reimplanting the cell and/or progeny of the second cell into a female which carries the resulting embryo to term.

36. The method according to claim 34 or 35, wherein the animal is a transgenic animal.

37. The method according to claim 34 or 35, wherein the animal is a chimeric animal.

38. A method for introducing a modification of a target locus in a cell in a vertebrate, the method comprising:

introducing into a cell *ex vivo* a recombinant parvoviral vector that comprises:

a) a targeting construct which comprises a DNA sequence which is substantially identical to the target locus except for the modification being introduced; and

b) all or a portion of at least one parvoviral ITR or a functional equivalent;

wherein homologous pairing occurs between the targeting construct and the target locus resulting in the modification being introduced into the target locus; and

introducing the modified cell into a vertebrate.

39. The method of claim 38, wherein the modified cell is introduced into the same vertebrate from which the modified cell was obtained.

40. The method of claim 38, wherein the vertebrate is a mammal.

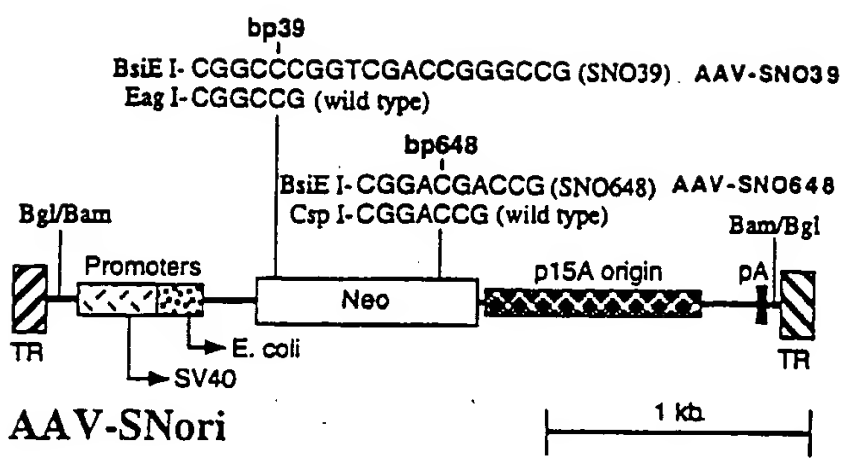
41. The method of claim 40, wherein the mammal is a human.

42. The method of claim 38, wherein the cell is a liver cell, a muscle cell, a fibroblast cell, a stromal cell, a skin cell, a stem cell, a hematopoietic cell, a fetal cord blood cell, a T-lymphocyte, a B-lymphocyte, or a monocyte.

43. A method for introducing a modification into a target locus in a cell in a vertebrate, the method comprising administering to the vertebrate a parvoviral vector having a recombinant viral genome comprising a targeting construct that comprises a DNA sequence that is substantially identical to the target locus except for the modification being introduced, wherein homologous pairing occurs between the targeting construct and the target locus resulting in the modifications being introduced into the target locus.

44. The method of claim 43, wherein the vertebrate is a mammal.

Figure 1A


bp39
BsiE I- CGGCCCGGTCGACCGGGCCG (SNO39) AAV-SNO39
Eag I-CGGCCG (wild type)
bp648
BsiE I-CGGACGACCG (SNO648) AAV-SNO648
Csp I-CGGACCG (wild type)
Bgl/Bam
Bam/Bgl
Promoters
Neo
p15A origin
pA
TR
E. coli
SV40
TR
AAV-SNori
1 kb

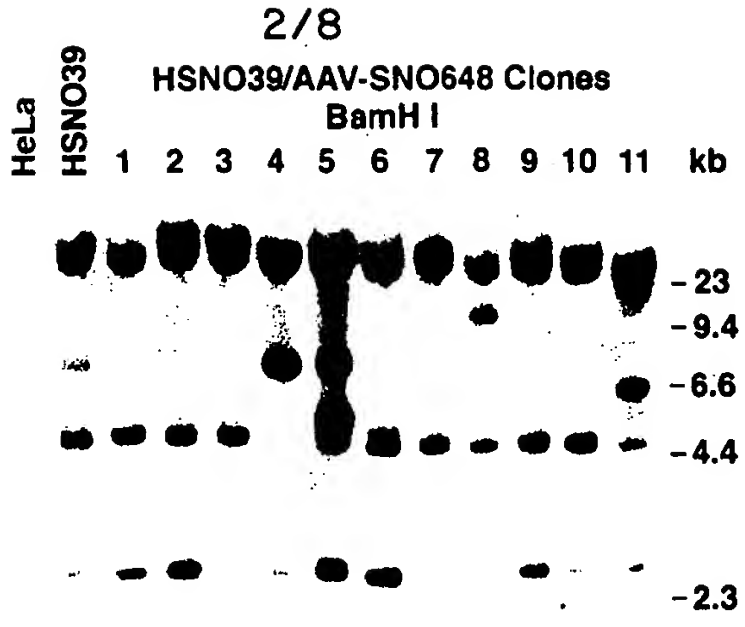


FIG. 1B.

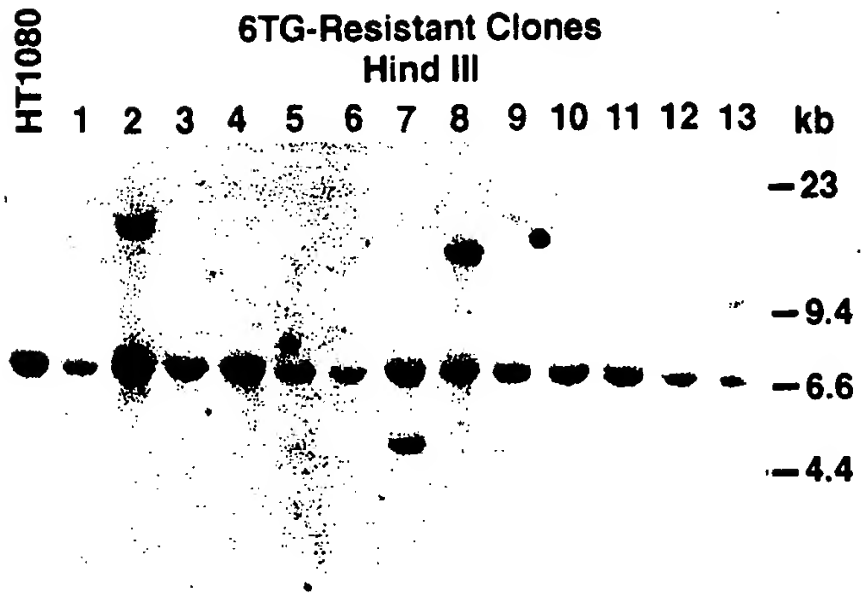


FIG. 2B.

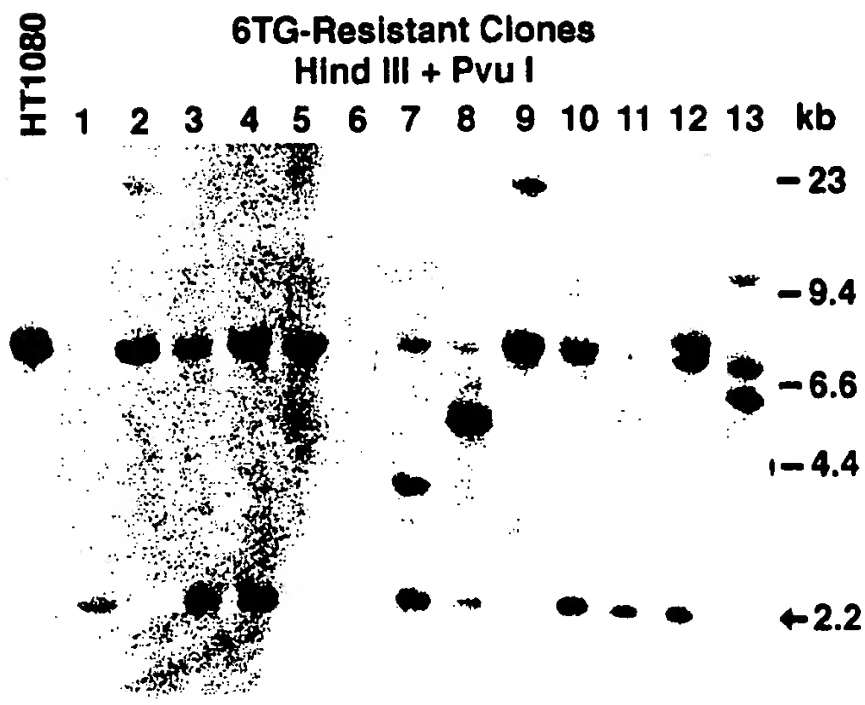


FIG. 2C.

Figure 2A

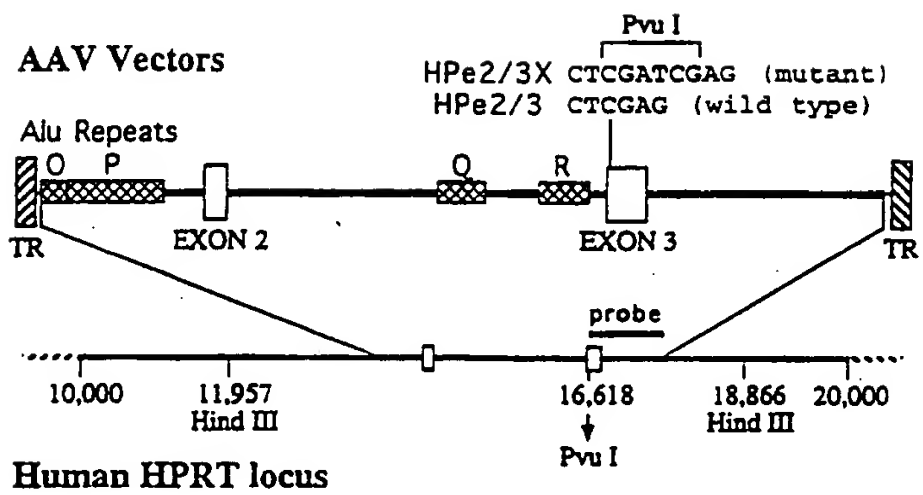

AAV Vectors
Pvu I
HPe2/3X CTCGATCGAG (mutant)
HPe2/3 CTCGAG (wild type)
Alu Repeats
O
P
Q
R
TR
EXON 2
EXON 3
TR
probe
10,000
11,957
Hind III
16,618
Pvu I
18,866
Hind III
20,000
Human HPRT locus

Figure 3

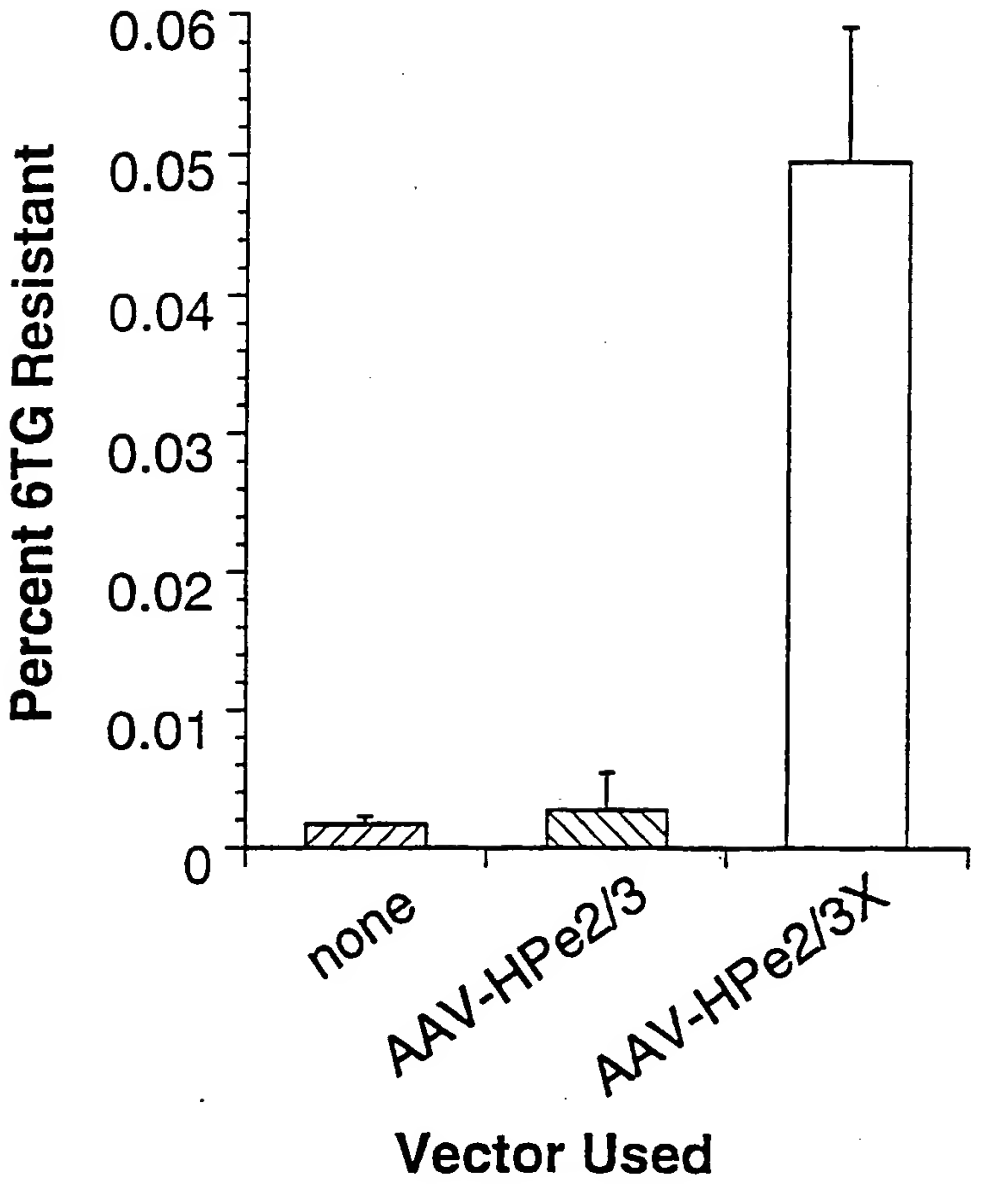

Percent 6TG Resistant
0.06
0.05
0.04
0.03
0.02
0.01
0
none
AAV-HPe2/3
AAV-HPe2/3X
Vector Used

Figure 4

Gene Correction in HSNO39 Cells by AAV-SNO648

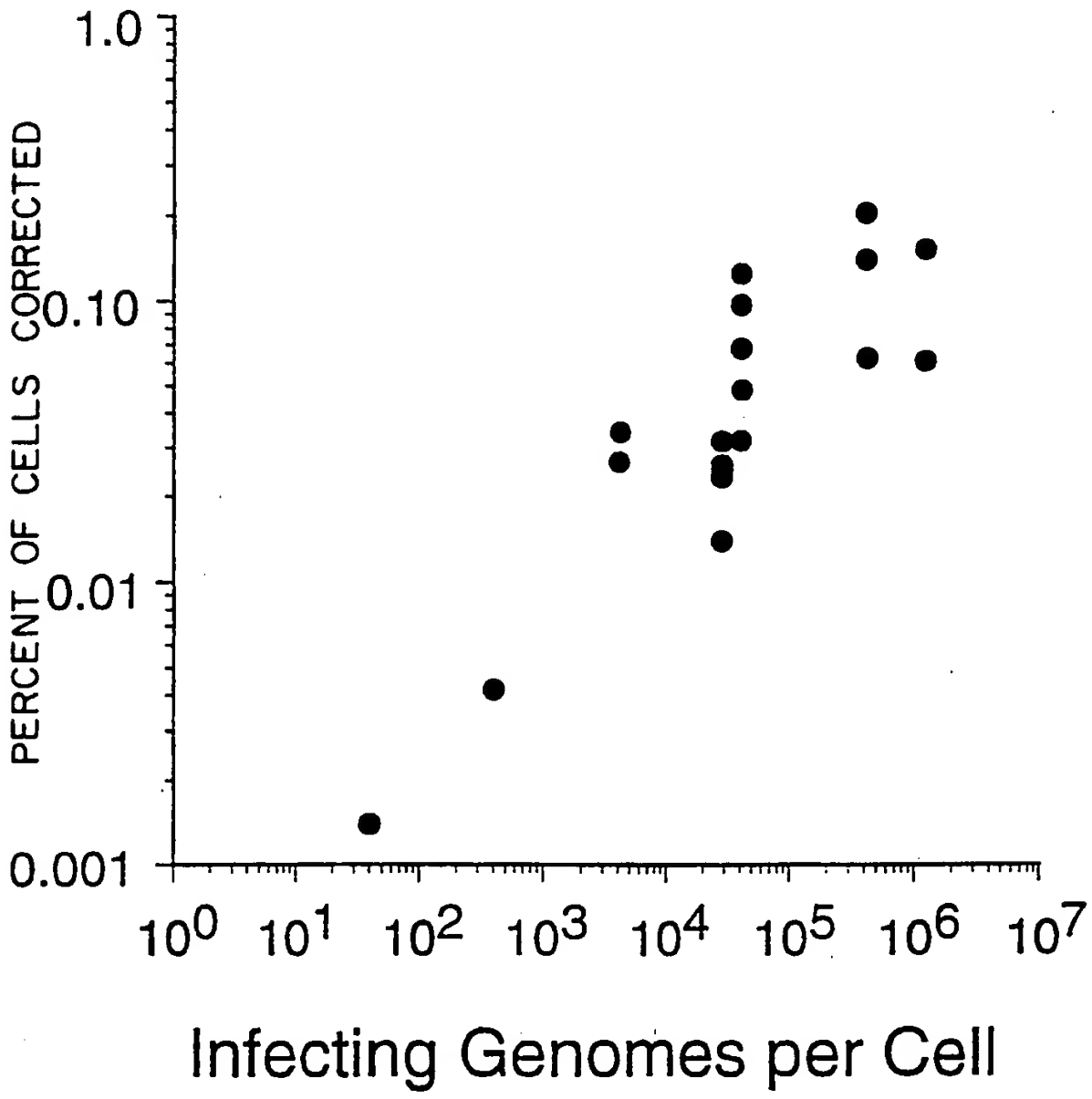


Figure 5

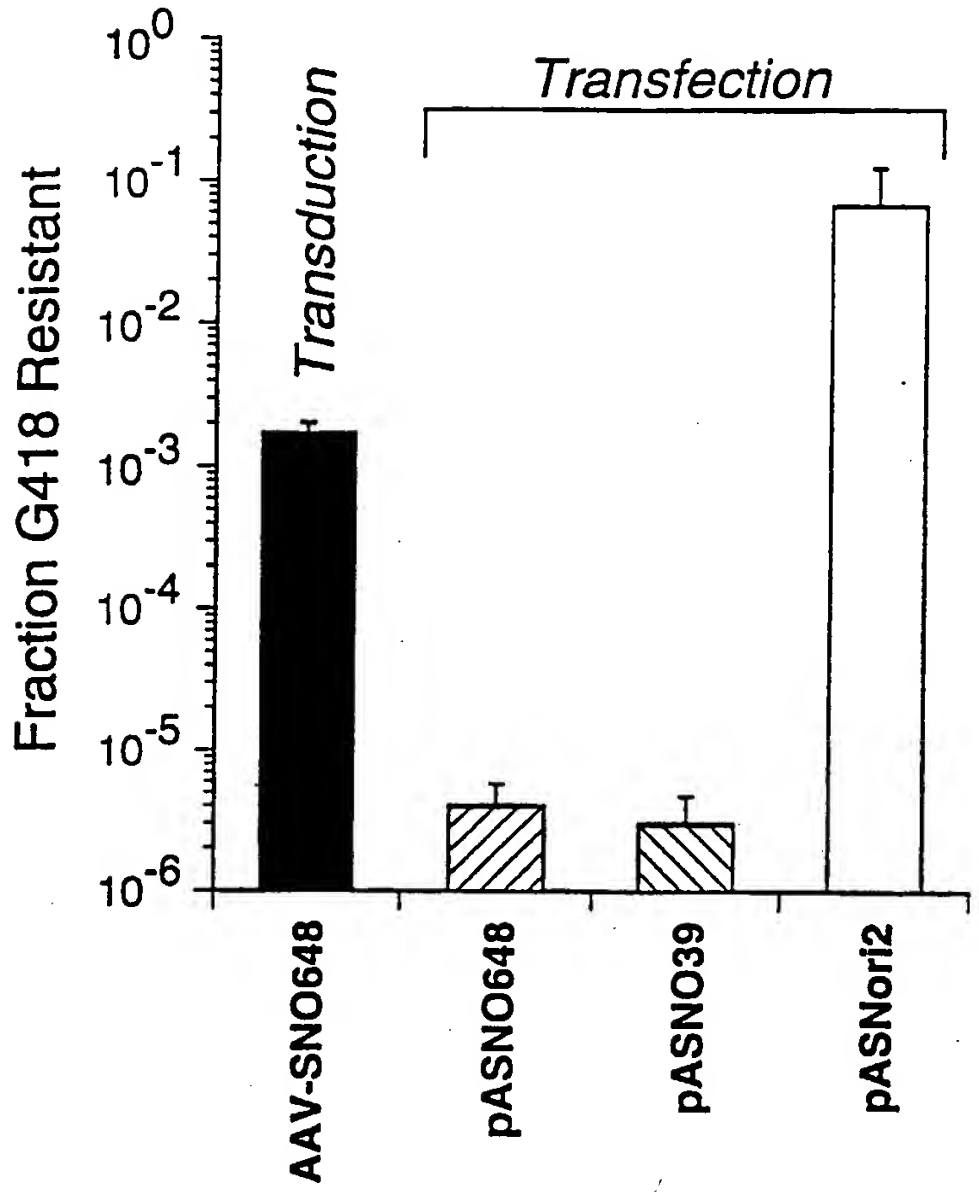

Transfection
Transduction
Fraction G418 Resistant
10^{0}
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
AAV-SNO648
pASNO648
pASNO39
pASNori2

Figure 6

HPRT Gene Modification in Normal Human Fibroblasts

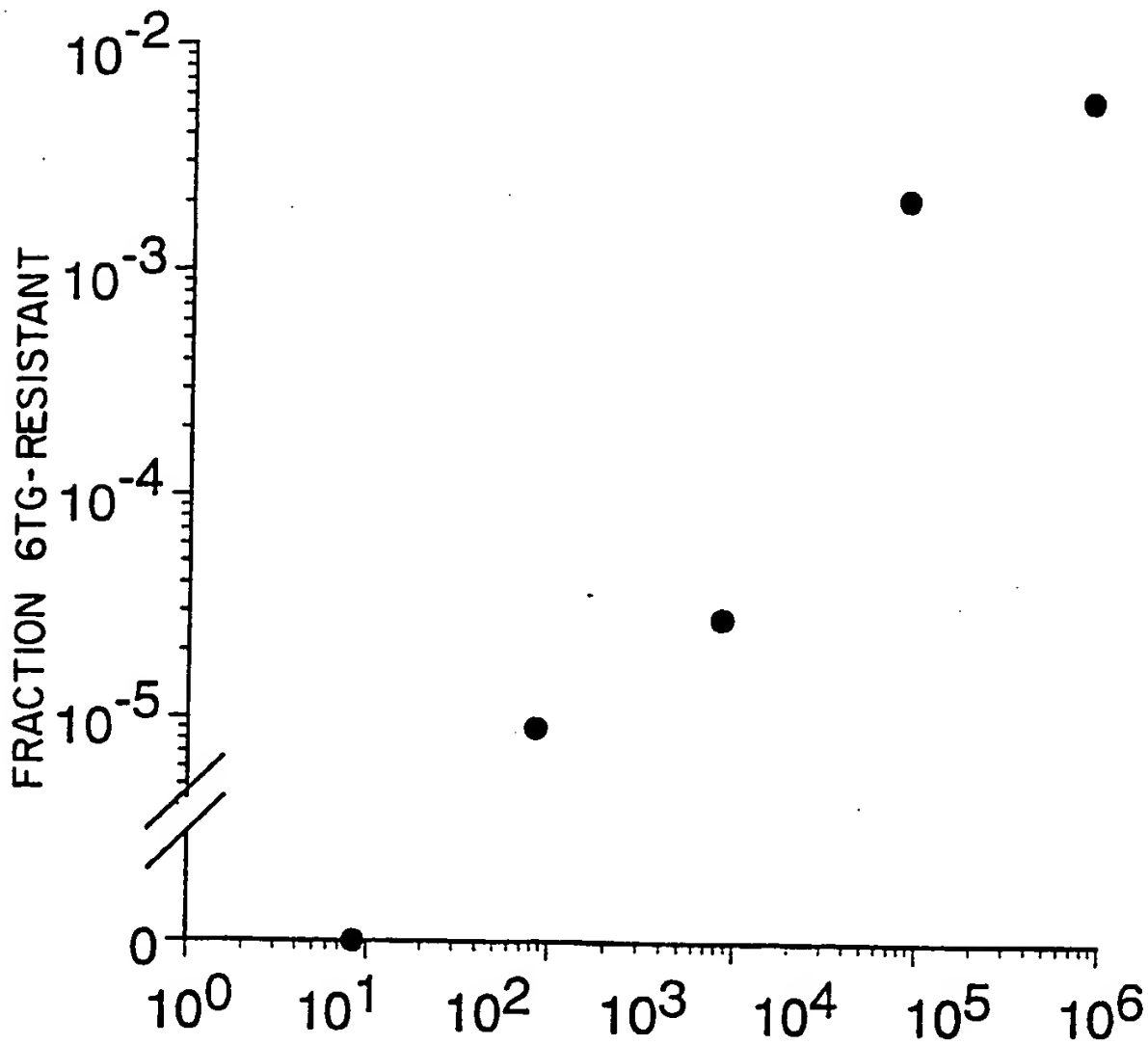
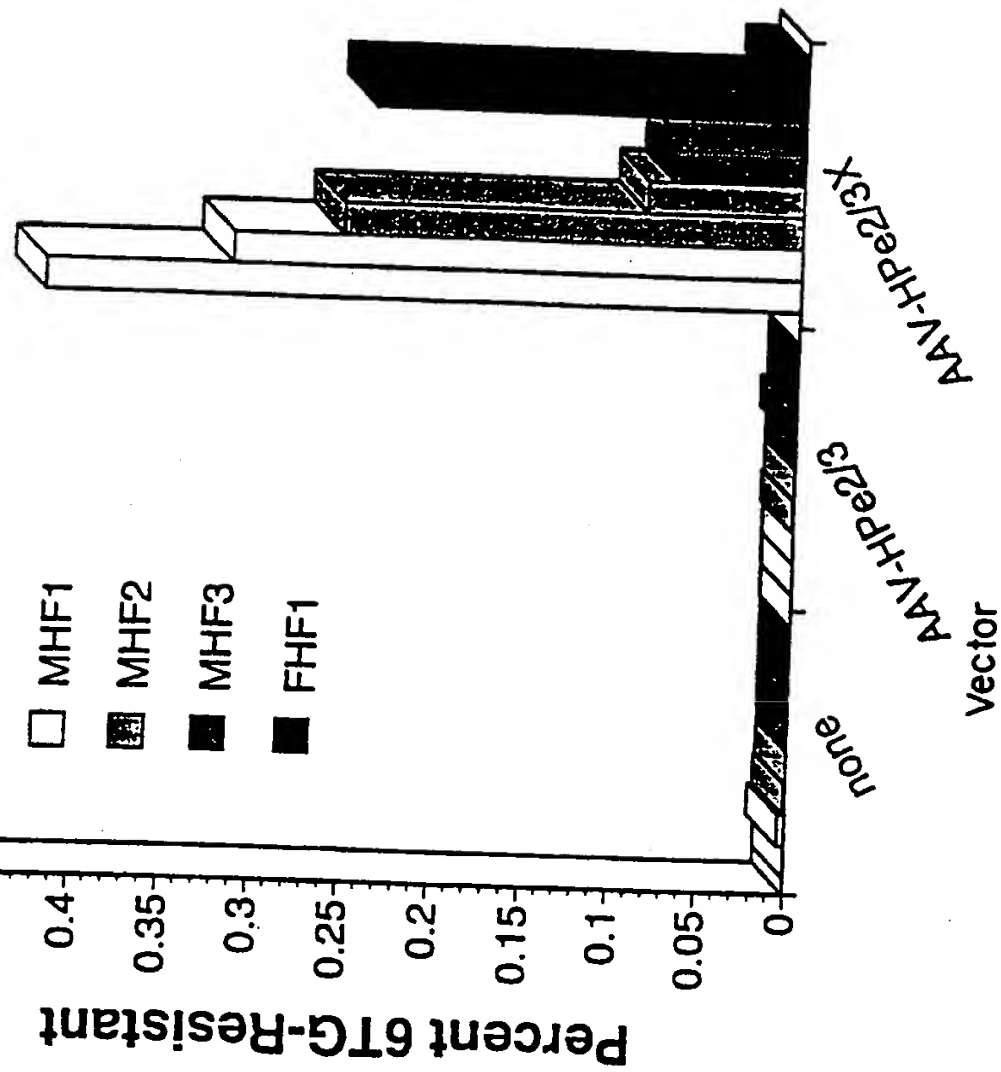


Figure 7

HPRT Gene Modification in Normal Human Fibroblast Lines



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/07964

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 5/10, 15/00, 15/10, 15/11, 15/12
US CL :435/172.3, 235.1, 243, 325, 410; 800/2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/172.3, 235.1, 243, 325, 410; 800/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,614,396 A (BRADLEY et al.) 25 March 1997, see entire document.	1-41
Y	US 5,602,307 A (BEAUDET et al.) 11 February 1997, see entire document.	1-41
Y	US 5,455,169 A (MULLAN) 03 October 1995, see entire document, especially col. 12, lines 6-8.	1-41
Y	BRADLEY, A. et al. Modifying the mouse: Design and Desire. Biotechnology, May 1992, Vol. 10, pages 534-539, see entire document.	1-41

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
30 JUNE 1998	10 AUG 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer BRIAN R. STANTON Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/07964

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

Databases:APS, Aidsline, biosis, agricola, anabstr, aquasci, biobusines, biotechds, ca, caba, cancerlit, caplus, ceaba, cen, cin, chacs, confsci, cropb, cropu, dgene, dissabs, drugb, druglaunch, drugu, drugu, embal, embase, fsta, genbank, healsafe, ifipat, jicst-eplus, medline

Search Terms:parvo?; adeno?; associat?; aav; homologous; recombination; hirata?/au; gene; target?; viral; vector#; embryonic; stem; virus; adenovirus; adenoviral; minute; identity; promot?; bradley?/au; method?;russell?/au